PHD

Mating system variation in relation to disease biology in Charadrius plovers (Alternative Format Thesis)

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Mating system variation in relation to disease biology in *Charadrius* plovers

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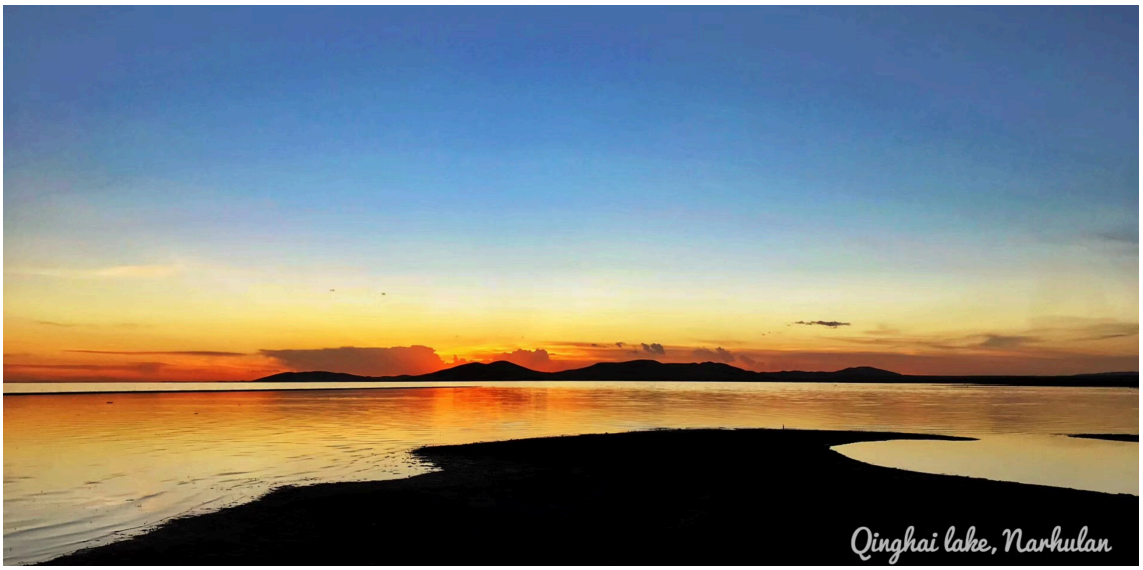
for my beloved family



Contents

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List of abbreviations

OSR: Operational sex ratio

ASR: Adult sex ratio

COVID-19: Coronavirus disease 2019

STIs: Sexually transmitted infections

VTIs: Vertically transmitted infections

GLMM: Generalized linear mixed model

GLM: Generalized linear model

LMM: Linear mixed model

SSD: Sexual size dimorphism

TMB: Template model builder

MCMCglmm: Markov chain Monte Carlo generalized linear mixed model

ANOVA: Analysis of variance

SE: Standard error

RDP: Ribosomal database program

OTU: Operational taxonomic units

NMDS: Non-multidimensional scaling

ANOSIM: Analysis of similarities

KP: Kentish plover

LSP: lesser sand plover

Summary

The study of social behaviours has been exuberant given the broad facets that it encompasses, from the social environment and sexual selection to mating behaviour and parental care. Social behaviours, especially mating and parenting behaviours, are highly variable and are associated with fitness-related processes in wild populations. An increasingly important element of social behaviour is the transmission dynamics of pathogens within and between social groups. Therefore, understanding how social behaviours impact the prevalence of disease in wild populations is an essential objective both in evolutionary biology and wildlife disease biology. The general objective of my dissertation is to explore the mating decision process and its implications in the prevalence of disease infections in *Charadrius* plover, a group of shorebirds with remarkable variation in breeding behaviours. Using empirical and theoretical modelling approaches, my PhD provided three novel insights. First, as evolutionary theory predicts that, in species that form pair-bonds, successful reproduction should lead to retention of the breeding pair for future reproduction. However, by investigating a polygamous plover population, I found the contrary since successful breeding promoted divorce, whereas failed breeding resulted in mate retention (Chapter 2). To check the generality of the latter results, I carried out a comparative study of 14 plover populations (Chapter 3): the result supported my previous finding, suggesting that plovers commonly benefit by mating with a new partner and reproduce shortly after divorce. Second, by using theoretical model, I showed how population sex ratio and disease characteristics drive the prevalence of sexually transmitted infections in males and females (Chapter 4). Third, I quantified composition of gut bacteria in two wild populations of plovers (Chapter 5) and showed that the gut microbial diversity is predicted by local environment rather than population social structures (i.e. age or sex of plovers). Taken together, my discoveries uncovered fundamental relationships between mating systems, population demography and disease prevalence using wild bird populations as model organisms. Given the significance of diseases in population demography of social human and non-human populations, further research needed to uncover how social structure impacts spread of diseases, and vice versa, how diseases impact on social structures.

Chapter 1 | Introduction

The evolution of social behaviour is of primary interest to scientists in the fields of behavioural ecology, evolutionary biology and conservation biology (Trivers 1985, Székely *et al*. 2010, Rubenstein and Alcock 2018). Social behaviour in humans and non-human animals focuses on interactions that occur between two or more individual of same species, it ranges from simple aggregations, communication across space to cooperation in sexual or parental behaviour, engaging in disputes over territory and access to mates. Among the many social behaviours, breeding behaviours (i.e. mating and parenting behaviours) stand out as having some of the most diverse features animals may exhibit (Andersson 1994, Székely *et al*. 2010, Davies *et al*. 2012). For instance, males courting and competing for access to females, or mates retaining each other for future partners; parental care includes the interactions between the caring male and female, as well as between the parents and the offspring (Royle *et al*. 2012). Much research has gone into understanding the proximate and ultimate causes of social behaviour (Székely *et al*. 2010, Davies *et al*. 2012, Rubenstein and Alcock 2018), and a recently accelerating research direction is to investigate social behaviour from diseases biology perspectives, since social interactions have implications for how diseases, parasites and infections spread through populations. For example, promiscuous sexual contacts between males and females, or parenting provisioning of offspring may all influence distribution, contamination and spread of pathogens and diseases in a population (Anderson and May 1991). Therefore, understanding the variation of social behaviours and its implication in disease in wild populations has great significance in evolutionary biology, wildlife disease biology and conservation biology (Blumstein *et al*. 2010, Traniello and Bakker 2020).

1.1 Breeding Systems

The concept of breeding system explores the relationship between mating behaviours and parental care in sexually reproducing animals, with the former encompassing some important facets of sexual selection - the manner of mate acquisition (e.g. courtship or competition), and mating system - number of mates acquired, and any forms of pair bonds (Reynolds 1996). The term breeding system is often used interchangeably with mating system (Darwin 1871, Emlen and Oring 1977), however, here I follow the definition from Reynolds (1996) that breeding system involves both mating behaviour and parental care by both sexes, including variation among individuals in extent of mate choice, form and duration of the pair bonds and parental care.

In most species, mating behaviour and parental care are different between males and females, where males tend to compete more intensely for access to mates and their gametes,

whereas females tend to engage more in parental care (Queller 1997, Parker and Pizzari 2015) but see (Clutton-Brock 2007). Trivers' theory (1972) suggested that such differences in breeding behaviours stem from anisogamy and parental investment. Since eggs are more costly than sperms and females typically bear the offspring and have a more limited reproductive rate than males. Therefore, female may prefer certain males for a variety of reasons to improve their reproductive success and offspring survival (Emlen and Oring 1977, Weatherhead and Robertson 1979). This results in sexual selection acting often more strongly on males, leading to the evolution of certain conspicuous physical traits (e.g. ornamentations, enhanced body size, weaponry such as antler and fangs etc.) that are used in competing for mates by force (*male-male competition*) or by charm (*female mate choice*) in males (Darwin 1871). Note that this does not mean that female-female competition or mate choice by males are unimportant; rather, it aims at characterising the "typical" patterns that suit some animal taxa better than others (Clutton-Brock 2007, Herridge *et al*. 2016).

There are different types of mating systems and parental care that exist in nature. The most general classification of mating systems following Klug's definition (Klug 2011) is monogamy (an exclusive pair-bonding between a single male and a single female within a reproductive bout or consecutive bouts or even lifetime; e.g. 90% of birds) and polygamy (males and/or females mate with multiple individuals within a reproductive bout). There are two classic polygamous mating systems: polygyny is when a male mates with more than one female, it is found in a few birds and insects but is most common in mammals (Thornhill and Alcock 1983, Clutton-Brock 1989); polyandry is when a female mates with more than one male, it can be found in insect, fish, mammals and birds (Kellogg *et al*. 1998, Eens and Pinxten 2000, Møller 2003, Gowaty 2012). The most common patterns of parental care are biparental care, which is commonly seen in monogamous populations; uniparental care, on the other hand, is common in polygamous populations (Kokko and Jennions 2008). Although these classifications refer to the general patterns observed in populations, it is worth mentioning that the association between mating system and parenting is often weak, in some polygamous species, both parents provide care (e.g. Northern lapwing *Vanellus vanellus*; Liker and Székely 1999). Traditional theory suggests that the difference between monogamous and polygamous populations will depend on the mate-monopolisation capacity which is dependent on variation in the spatial and temporal distribution of sexually active mates (or operational sex ratio, OSR, the ratio of sexually active males to females) and/or other resources (Emlen and Oring 1977).

However, the previous theoretical frameworks – such as Trivers (1972) and Emlen & Oring (1977) – have shortcomings. For example, Trivers' theory (1972) that females provide more parental care than males is because females have greater pre-mating investments than male, which they cannot stand to lose. This, however, commits the "Concorde Fallacy" as optimal decisions should depend on future payoffs instead of past costs (Kokko and Jennions 2008). In addition, given each offspring has precisely two genetic parents (the Fisher condition, McNamara *et al*. 2000), a

biased OSR generates frequency-dependent selection, which favours increased parental investment by whichever sex faces more intense competition (Kokko and Jennions 2008). Moreover, a comparative study on shorebirds shows that offspring development constrains the evolution of both parental care and mate competition, which is less consistent with Trivers' parental investment theory (Thomas and Székely 2005). Second, the OSR as an empirical measurement of sexual selection has limitations (Kokko *et al*. 2012). For example, OSR lacks generality and stability as it inevitably changes with the synchrony of female sexual activity. Moreover, studies have shown that OSR has no effect (Klug *et al*. 2010, Jennions *et al*. 2012) or a negative effect (Fitze and Le Galliard 2008, 2011) on sexual selection. More recent studies have addressed the importance of adult sex ratio (ASR) in breeding system variation (Kokko and Jennions 2008, Székely *et al*. 2014, Carmona-Isunza *et al*. 2015, Eberhart-Phillips *et al*. 2018). ASR is related to a broader aspect of life history such as individual mortality, maturation and movement patterns (Veran and Beissinger 2009, Kosztolányi *et al*. 2011), providing us a wider scope for investigating breeding system evolution. Finally, subsequent studies that built on Trivers' theory often analysed mating behaviours, mating opportunities and parental care in isolation from each other, as they assumed that parental investment, OSR or the mating system are fixed in a population (Emlen and Oring 1977, Maynard Smith 1977). The potential feedback among those aspect of reproduction is overlooked.

Following game-theoretic models (Maynard Smith 1977, McNamara *et al*. 2000), a conceptual framework was proposed to illustrate the feedback relationships between mating behaviours and parental care, suggesting that mating opportunity, mating and parenting behaviours are interrelated (Figure 1.1; Székely *et al*. 2000). The variation in mating opportunities will alter the availability of partners, it leads unmated individuals to accept or reject a mate, and leads mated individuals to care for offspring or desert them. The differences in mating and parental care strategies among individuals generate different distribution of mating patterns (e.g. monogamy, polygyny, polyandry) and parental care patterns (e.g. biparental care, female-only care, male-only care). This theoretical framework allows us to understand breeding systems based on the entire adult populations, not only focusing on breeding individuals, but also including those individuals that are not breeding at any given time.

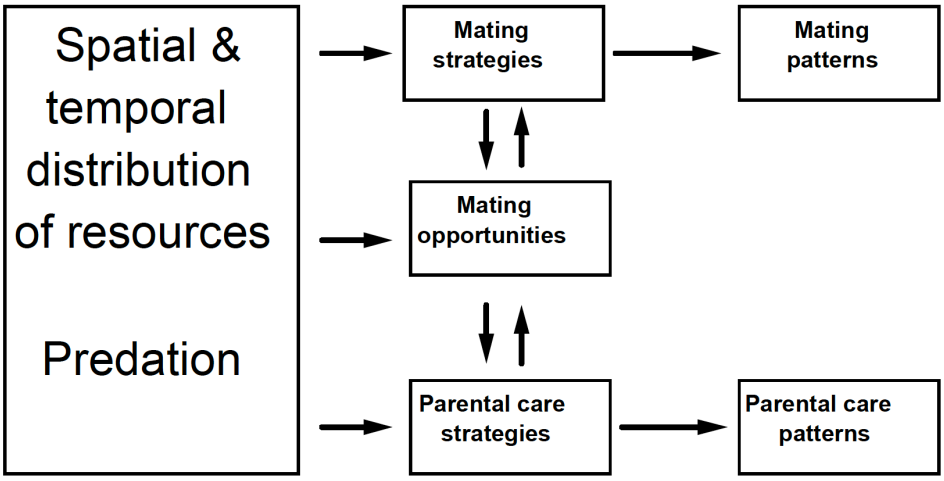

Figure 1.1 The conceptual framework illustrating the relationship between mating behaviours and parental care. Adapted from Székely *et al.* 2000.

1.1.1 Mate fidelity and divorce

Mating strategy is an important aspect of breeding system study framework (Figure 1.1), it describes the mating decisions that individuals adopt for the purpose of improving reproductive success, and are important in structuring animal mating systems (Neff and Pitcher 2005, Székely *et al*. 2006, Székely *et al*. 2014). In species that form pair-bonds (e.g. birds, primates), fidelity to a mate or divorce for several breeding events varies widely among species with some species showing long-term mate fidelity or even life-time mate fidelity until one partner dies (Black 2001, Reichard and Boesch 2003). Other species, however, exhibit short-term mate fidelity, in which an individual terminates the relationship at the end of one breeding attempt and initiates another breeding with a new mate while the old partner is still alive. Why do some individuals adopt short-term mate fidelity, while others pair for life? Here I briefly conclude three major factors that explain mating decisions in a context of costs and benefits according to previous studies, although the importance of these factors most likely varies among animal taxa.

(i) *Ecological constraints*. Ecological constraints determine the availability of resource, such as food availability and habitat quality, that an individual can get to improve the survival of offspring. Variations and stochasticity in resource such as food availability and habitat quality may affect the reproductive benefits of different mating decisions (Gilburn and Day 1994, Blomqvist *et al*. 2001, Welch 2003, Candolin *et al*. 2007, Eberhart-Phillips 2019). For example, in territorial birds, an individual divorces a mate to find a more preferable alternative mate with better resource (Ens *et al*. 1993); in marine fishes, the pair stay together to defend feed territories (Whiteman and Côté 2004).

(ii) *Time constraints.* One aspect of time constraints is often related to a series of life history traits (e.g. life span, survival and mortality rates, body size, age etc; Andersson 1994, Choudhury

1995, Black 1996, Wheelwright and Teplitsky 2017), because the confounding effect of these life history trait may determine the breeding potential of an individual, thus different individuals may benefit from different mating decisions. Another aspect of time constraint is related to abiotic factors such as temperature and precipitation, which play important roles in altering the duration of suitable breeding periods, therefore limiting or promoting mate fidelity. For example, a prolonged breeding period allows individual to have multiple breeding events with same or different mates (Botero and Rubenstein 2012, Eberhart-Phillips 2019).

(iii) *Mating opportunity constraints*. Mating opportunity can be estimated by ASR (Székely *et al*. 1999). Variation in ASR leads to different mating opportunities among populations, therefore, divorce maybe favoured in populations with biased ASR and less prevail in populations with even ASR (Székely *et al*. 1996, Parra *et al*. 2014). Mating opportunity may also relate to parental care, as care by one sex can affect mate availability and the likelihood that the non-caring sex will be able to find a mate (Queller 1997; Kokko & Jennions 2008).

Despite the considerable number of studies of mating systems in a wide range of taxa, our understanding of variation in mating decisions is still limited. First, the studies of mating decisions mostly focus on single populations /species, therefore, the generality and stability of current theories/hypotheses remain to be tested, comparative studies are essential to understand the mechanism of different mating decisions. Second, more empirical studies are needed to understand how the aspects of ecology and life history influence mating decisions. Third, the quantitative measures of mating systems vary widely among studies, which undermines the reliability and commonality of the results. Understanding the mating decision processes will not only better enable us to understand the evolutionary feedback within the components of the breeding system study framework (Figure 1.1), but also will provide us a better scope to linking breeding behaviours to other biological and ecological processes.

1.2 Breeding System and Disease Biology

Animal populations show considerable heterogeneity in social behaviours (including mating behaviours and parental care). Variation in social behaviours determines how individuals assess resources and interact with other social group members, therefore, the patterns of interaction should predict prevalence rates in a population, and via dispersal of individuals, influence global dispersal of pathogens (Krause *et al*. 2007). A better understanding of how social behaviour shapes infectious disease transmission is important for public health, conservation biology and the world economy (Kappeler *et al*. 2015). This is exemplified by the recent COVID-19 pandemic sweeping the globe, causing morbidity, mortality, and economic loss. Here we focus on the role of mating behaviours and sex ratio variation on disease transmission.

1.2.1 Mating system and disease transmission

Mating systems reflect different patterns of social contacts (e.g. mate acquisition, copulations and breeding dispersal) between a subset of individuals within a population (Cross and Parker 2004). The differences in social contact patterns may result in different infectious disease transmission dynamics, including sexually transmitted infections (STIs) and non-STIs (e.g. airborne pathogens that are transmitted via close contact during copulations). For example, in social animals, the contact network between individuals are more interconnected in polygamous populations than in monogamous populations (Cunningham *et al*. 2018), which reflects on more intense mate acquisition (i.e. male-male competition and lek behaviour) and sexual contacts (e.g. alpha male copulates with more females than other males in the population) in polygamous populations than monogamous populations, generally leading to increased pathogen transmission (Kokko *et al*. 2002, Nahrung and Clarke 2007, Ashby and Gupta 2013). In solitary sexual species, the social contacts among individuals are far less frequent than social species, however, individuals must come together for reproduction, where potential infectious disease transmission takes place (Dröscher and Kappeler 2013, Kappeler *et al*. 2015). Disease prevalence dynamic is not only influenced by sexual behaviours, but also special dispersal of individuals. For example, a monogamous pair that holds a territory interacts mostly with close neighbours, therefore, the chance of infections is lower than promiscuous species where males and/or females disperse in a wide range of territories to seek mates.

Beyond the literature on STIs (Thrall *et al*. 1997, Thrall *et al*. 2000, Ashby and Gupta 2013), there are few examples of the protective role of social relationships in the epidemiology of infectious diseases (Bonds *et al*. 2005, Prado *et al*. 2009). The consensus of the theoretical works on STIs and mating systems has been that, within a population, the sex with the higher variance in mating success should have lower prevalence of infection. For instance, in most mammals, due to the male-male competition for mates, males usually have higher variance in mating success, thus, sexually active males (polygamous males) are often more likely to contract and transmit STIs than monogamous females (Kokko *et al*. 2002, Ashby and Gupta 2013), as a result, the STIs show a female-biased epidemic (Ashby and Gupta 2013, Nahrung and Clarke 2007). Other theoretical works looked into the evolutionary relationships between mating system and STIs. They predict that STIs generate selection pressure for monogamy, because while individuals may benefit from mating with multiple partners to improve breeding success, they also risk contracting infections when mating. STIs may therefore lead to a reduction in mating skew towards monogamy (Sheldon 1993, Loehle 1995, Poiani and Wilks 2000), although in some cases STIs may select against monogamy (McLeod and Day 2014).

However, current studies on the relationship between mating systems and disease transmission have several limitations: first, much of the research is more heavily focused on STIs than non-STIs. Second, studies of STIs are dominated by theoretical modelling approaches, yet the empirical studies focus on human populations with fewer empirical studies in animal populations

(Eames and Keeling 2002, Panchanadeswaran *et al*. 2006, de Coul *et al*. 2014). Third, much of the work overlooked the other factors that may relate to disease transmission dynamics. For example, sex ratio may influence epidemiology by affecting mating dynamics (Rankin and Kokko 2007), and pathogen characteristics (e.g. transmissibility, virulence) may differ between males and females due to physiological or behavioural difference (Moore and Wilson 2002, Miller *et al*. 2007).

1.2.2 Parental care and disease transmission

Parental care is another aspect of social behaviour that is closely related to the transmission of pathogens. A vertically transmitted infection (VTIs), defined as an infection that is transferred from mother to embryo, fetus, or new-born before, during, or shortly after parturition (Maclachlan and Dubovi 2010, Chakarov *et al*. 2015), is a common pathogen transmission path in a range of taxa (Potti *et al*. 2013, Arora *et al*. 2017, Lowe *et al*. 2018). For example, studies in mammalian species show that virus infections are transmitted from parent to offspring during embryological development, during the actual process of birth and after birth (reviewed by Mims 1981). It is similar in birds; bacterial, viral and parasitic diseases can be vertically transmitted from an infected female through the egg to the embryo or progenies (Cobb 2011, Dona *et al*. 2017).

Existing studies of parental care and disease transmission are limited to VTIs, as patterns of parental care are widespread and highly diverse across species, various paths of transmission relating to parenting behaviours should exist and remain to be tested. For example, in species with biparental care, the pathogen may not only transmit from the female to the offspring before, during, or shortly after parturition, but also transmits from the feeding by the male. Some species even demonstrate cooperative breeding where more than two individuals contribute to a single brood of young (Cockburn 2006), so it is certain that some individuals cannot be parents of some of the young they are rearing, which might also enhance the risk of infections to the young. In addition, in avian species, altricial chicks are mainly fed by the parent(s), whereas precocial chicks feed themselves; altricial chicks might be more likely contract infections from parent(s) than precocial chicks. Such work will enable us better to understand how parental care influences disease transmission, and it is important to unveil the interactions between breeding system and disease biology in wild populations.

1.2.3 Gut microbiota

Animals harbour an extensive, dynamic microbial ecosystem in their gut and almost any other parts within or on their bodies (Münger *et al*. 2018). Interactions between hosts and microbiota influence various aspects of host functions, including development, fecundity, metabolism, immunity (Shapira 2016). On the one hand, with the advancement of the next-generation sequencing methods, more and more evidence indicate that the microbiota can also have important consequences on social behaviours, for instance aggregations (Wada-Katsumata *et al*. 2015), mate choice (Sharon *et*

al. 2013, Theis *et al*. 2013), or kin discrimination (Matsuura 2001). On the other hand, social contact is another mechanism that can mediate the acquisition and flow of microbiomes among individuals (Song *et al*. 2013). For example, in humans, gut microbiota establishment of infants is initially acquired from the mothers (Nuriel-Ohayon *et al*. 2016); in primates, individuals from the same community have more similar microbial consortia than do individuals from different communities (Degnan *et al*. 2012); moreover, a benefit of social living in many species may be the transmission of beneficial microbes (Lombardo 2008). How these complex host-microbe interactions are mediated is an open question.

Among the microbiota, gut microbiota (defined as a diverse community of bacteria that reside within the gastrointestinal tract) stands out as a particular field as the highly specialised intestinal environment poses greater selective pressure on microbial communities, and its implication for nutrient digestion and uptake, detoxification, immune system development, thus influencing the physiology, behaviour, and fitness of the host (Ley *et al*. 2008, Kohl 2012, Waite and Taylor 2015). Research has shown great diversity in gut microbial community among taxa (Figure 1.2; Kostic *et al*. 2013), and such diversity is shaped by various factors, including environment (e.g. food intake, habitat; Hird *et al*. 2014), physiology (Ellis *et al*. 2013), phylogeny (Ley *et al*. 2008, Goodrich *et al*. 2014), and behaviours (e.g. migration and social interactions; Bisson *et al*. 2009, Leftwich *et al*. 2018). However, microbiology of the avian gastrointestinal tract is a relatively new field, and is still in its infancy compared to mammalian and human studies (Grond *et al*. 2018). Understanding the gut microbiota in avian species is important for several reasons. First of all, as is mentioned above, research on gut microbiota of birds has lagged behind other taxa, most of which has heavily focused on domestic poultry (Sheldon 1993, Lockhart *et al*. 1996). Second, birds commonly demonstrate complicated social behaviours, therefore, understanding gut microbiota in birds will help us understand the host-microbe interactions in avian species. Third, gut microbiota in avian species play an important role in the context of disease prevalence. For example, pathogenic microbes have been detected residing in bird cloacal cavities (Rowe *et al*. 2020). Given the dual function of bird cloacal as the chamber for gamete transfer and excretion (Sheldon 1993), it provides a potential social-behaviour-based transmission route for pathogenic microbes in avian gut microbiota (Azambuja *et al*. 2005, Tsiodras *et al*. 2008).

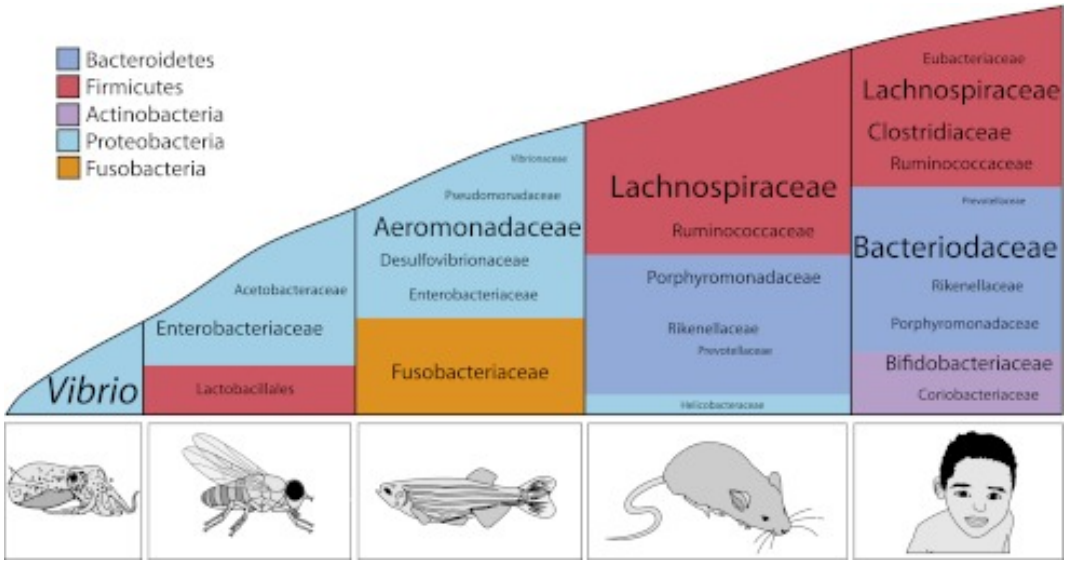

Figure 1.2. The structure of the gut microbiota across species. Although there can be significant interindividual variation in the composition of the microbiota, broad trends exist within a given species, particularly at the phylum level. Phyla are represented by colour, and the relative abundance of the lower taxonomic levels is indicated by font size. Adapted from Kostic *et al.* (2013).

1.3 Plovers as Ecological Model Species

Shorebirds are a group of charismatic animals that vary widely in life-history characteristics and social behaviours, which make them ideal organisms to study social organisations and address various questions in evolutionary ecology and behaviour (Székely and Reynolds 1995, Thomas *et al*. 2007, Colwell and Haig 2019). In this dissertation I use plovers of the genus *Charadrius*, within the Charadriiformes (shorebirds, gulls, alcids and allies) as focal organisms. *Charadrius* consists of 30 species of small plovers that breed on all continents (dos Remedios *et al*. 2015). I used the following *Charadrius* species for different research in this thesis: snowy plover (*Charadrius nivosus*), Kentish plover (*Charadrius alexandrinus;* Figure 1.3), lesser sand plover (*Charadrius mongolus schaeferi*), white-fronted plover (*Charadrius marginatus*), piping plover (*Charadrius melodus*), red-capped plover (*Charadrius ruficapillus*), two-banded plover (*Charadrius falklandicus*), killdeer (*Charadrius vociferus*) and Malaysian plover (*Charadrius peronii*). All these plover species provide an excellent model system to study breeding system evolution for three reasons. First, all species chosen are closely related (dos Remedios *et al*. 2015) and exhibit similar life-histories and ecology. Second, there is ample breeding system variation among plovers that ranges from monogamous with biparental care to polygamous with male-only care. Third, high quality breeding data is available for several plover species since several populations have been studied in detail for up to nine years using the same standard methodologies (Székely 2019).

Figure 1.3. A male Kentish plover (left) and an incubating female Kentish plover at Bohai Bay, China. Photos from Pinjia Que.

1.4 Dissertation Outline

There are two major objectives in this dissertation. First, I focus on the mating behavioural component of breeding system, aiming to investigate the mating decision patterns in several *Charadrius* populations, and provide empirical evidence to the relationships between mating decisions and life history traits, social and ecological environmental predictors. Second, I explore the implications of mating behaviour in the prevalence of disease risks and infections.

For the first objective, I present two closely related studies. In Chapter 2, I investigate mate fidelity in a sequential polygamous shorebird, the snowy plover. Using 6 years of data from a well-monitored population in Bahía de Ceuta, Mexico, I investigate predictors and fitness implications of mate fidelity both within and between years. As a follow-up study, in Chapter 3, I test whether the mating strategies in snowy plovers are consistent across plover populations that breed in ecologically different environments. I explore the life history traits, social and ecological environmental predictors that are related to mating decisions, by using 73 years of data from 14 well-monitored plover populations worldwide.

To address the second objective, I implement a theoretical analysis and an empirical study. Chapter 4 focuses on the interaction between host mating system and sexually transmitted diseases. By using a mathematical model, I examine the effects of ASR variation and disease characteristics (i.e. transmission rate and virulence) for STI epidemiological dynamics. To follow up the theoretical predictions, I present the results of a gut microbiome study (Chapter 5) where I investigate the gut microbial community using bacterial DNA sequences from two *Charadrius* populations (Kentish plover and lesser sand plover) that breed in Tibetan plateau. Finally, in Chapter 6, I conclude my dissertation with a synthesis of my review and discussion of my empirical results. I summarise the novel results of my dissertation research and identify useful areas for future research.

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Chapter 2 | Mate fidelity in a polygamous shorebird, the snowy plover (*Charadrius nivosus*)

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Authors' Contributions

N. H., P. H. and T. S. conceived the project;

K. K., M. A. S.-M. and M. C.-L. provided the data;

N. H. and K. K. carried out the statistical analyses.

All authors contributed critically to the drafts and gave final approval for publication.

Abstract

Social monogamy has evolved multiple times and is particularly common in birds. However, it is not well understood why some species live in long-lasting monogamous partnerships while others change mates between breeding attempts. Here, we investigate mate fidelity in a sequential polygamous shorebird, the snowy plover (*Charadrius nivosus*), a species in which both males and females may have several breeding attempts within a breeding season with the same or different mates. Using six years of data from a well-monitored population in Bahía de Ceuta, Mexico, we investigated predictors and fitness implications of mate fidelity both within and between years. We show that in order to maximize reproductive success and account for time constraints within a season, individuals divorce after successful nesting and re-mate with the same partner after nest failure. Therefore, divorced plovers, counterintuitively, achieve higher reproductive success than individuals that retain their mate. We also show that different mating decisions between sexes predict different breeding dispersal patterns. Taken together, our findings imply that divorce is an adaptive strategy to improve reproductive success in unpredictable environments. Understanding mate fidelity is important for the evolution of monogamy and polygamy, and these mating behaviours have implications for reproductive success and population productivity.

Keywords

mate fidelity, divorce, polygamous, nesting success, breeding dispersal, *Charadrius nivosus*

2.1 Introduction

The decision of retaining a mate for several breeding events or divorcing, is a key element of reproductive decisions in several species, as it can affect reproductive success and subsequent survival of the parents (Neff and Pitcher 2005, Székely *et al*. 2006, Culina *et al*. 2014, Székely *et al*. 2014). Social monogamy, defined as a system where an adult has only one social partner of the opposite sex at a given time or throughout a time period, is commonly observed in birds, but also occurs in invertebrates, fish, amphibians, reptiles, and mammals (Møller 2003, Lukas and Clutton-Brock 2013). Social monogamy partnerships are highly variable in terms of duration. Some species show long-term mate fidelity or even life-time mate fidelity until one partner dies (Black 2001, Reichard and Boesch 2003). Other species, however, exhibit short-term mate fidelity, in which an individual terminates the relationship at the end of one breeding attempt and initiate another breeding with a new mate while the old partner is still alive (termed, sequential polygamy). Why do males and females adopt short-term mate fidelity, whilst others pair for life?

Several hypotheses have been put forward emphasizing the impact of either breeding time-constraints (or breeding success) on mate fidelity or divorce in socially monogamous species. On the one hand, retaining a mate reduces the time and energy costs of searching for a new mate therefore facilitate a fast re-mating ("fast-track hypothesis"; Adkins-Regan and Tomaszycki 2007, Perfito *et al*. 2007). Retaining a mate also enhance breeding performance thereby improving reproductive success (Gabriel and Black 2013). In addition, successful breeding may also facilitate retaining the mate for future breeding (Black 2001, Flodin and Blomqvist 2012). On the other hand, changing a mate may be beneficial in long-lived species, individuals divorce their partner to mate with good quality partners in order to improve breeding success ("incompatibility hypothesis"; Coulson 1966, Kempenaers *et al*. 1998). In species with short life span (or short breeding season), individuals improve reproductive success by mating with multiple mates to make the most out of limited time ("extra-pair mating hypothesis"; Birkhead and Fletcher 1992, Arnqvist and Nilsson 2000).

Mating decisions may be related to breeding dispersal – the latter defined here as the movement of an adult from one breeding location to another within or between years (Greenwood 1980, Clobert *et al*. 2001). On the one hand, breeding dispersal may differ between the sexes in response to sex differences in mating strategies since the more polygamous sex is expected to disperse farther to find new mating partners (Greenwood 1980, Trochet *et al*. 2016, D'Urban Jackson *et al*. 2017, Székely 2019). On the other hand, mate fidelity can be viewed as a by-product of site fidelity in some species (Morse and Kress 1984, Bried *et al*. 2003), whereas changing the nest site would lead to mate change in some other species (Pietz and Parmelee 1994, Thibault 1994).

A further factor that may influence mate fidelity is re-mating opportunity. In species or populations with a biased adult sex ratio, divorce is commonly initiated by the rare sex since the rare sex has higher mate availability than the common sex (Liker *et al*. 2014, Parra *et al*. 2014). For

example, experimental studies of species with biased adult sex ratio showed that by experimentally creating unmated males and females, re-mating times were shorter for rare sex than for common sex (Székely *et al*. 1999, Parra *et al*. 2014).

Nevertheless, studies of mate fidelity tended to focus on monogamous species across breeding years, yielding different adaptive implications of mate fidelity (Dubois and Cézilly 2002, Bried *et al*. 2003). Monogamous systems are generally characterized by high level of breeding philopatry (Moore and Ali 1984, Saalfeld and Lanctot 2015) and/or biparental care of the young (Eberhart-Phillips *et al*. 2018), features that tend to promote mate fidelity. However, the causes and fitness implications of mate fidelity in sequential polygamous species that exhibit variable duration of pair bonds (e.g. within a breeding year), different levels of philopatry or breeding dispersal are still poorly understood.

Here, we investigate potential predictors and fitness implications of mate fidelity in a sequential polygamous shorebird, the snowy plover (*Charadrius nivosus*), a ground-nesting, near threatened shorebird distributed on sparsely vegetated coasts and alkaline lakeshores across the temperate and tropical regions of the Americas. They typically lay a 3-egg clutch with both parents providing care during the incubation stage, chicks are precocial and nidifugous, which only require uniparental care (usually males) during brood-rearing (del Hoyo *et al*. 2018). This species is an ideal model for investigating mate fidelity: they have a flexible mating system, both males and females may have several mates sequentially in a single breeding season up to four breeding attempts (Page *et al*. 2009). It is typically females that mate with more partners than males do, since females tend to desert their broods soon after hatching, and leave the males to look after the young until independence (Warriner *et al*. 1986, Carmona-Isunza *et al*. 2015). Female desertion has been linked to male-biased adult sex ratio (ASR): 0.53 (proportion of males in the adult population) was estimated by Stenzel *et al*. (2011) based on adult survival, whereas more recent estimate that took into account hatchling sex ratios, chick survival and adult survival estimated a strongly male-biased ASR (0.638, Eberhart-Phillips *et al*. 2018). Snowy plovers may still retain their mate between clutches within or between years. Furthermore, a recent paternity analyses showed low rates (less than 5%) of extra-pair paternity in the snowy plover so that social pairs are a good proxy for genetic relationships and thus reflect Darwinian fitness (Maher *et al*. 2017).

Using snowy plovers as a model organism, here we investigate whether mate fidelity (or divorce) is an adaptive strategy that maximizes reproductive success in a species with limited breeding period (Choudhury 1995, Plaschke *et al*. 2019). We focus on three main aspects of mate fidelity. First, we investigate patterns of mate fidelity both within and between years in both males and females. Second, we explore if previous nesting success predict mate retention (or divorce) by males and females both within and between years. Finally, we investigate mate fidelity in relation to breeding dispersal and re-mating time: (i) whether breeding dispersal is related to mate fidelity

both within and between years; (ii) whether the re-mating time may differ between divorced and retained mates within years.

2.2 Methods

2.2.1 Study site and field methods

The present study was conducted at Bahía de Ceuta, Sinaloa, Mexico (23° 54′ N, 106° 57′ W). In this population, snowy plovers nest on extensive saline ponds and saltpans (Carmona-Isunza *et al*. 2015). The breeding season generally occurs from mid-April to mid-July, with 30–100 breeding pairs every year. Breeding data were collected from 2006 to 2011 (n = 625 nests). Data collection in the field followed the methods of Székely *et al.* (2008). Briefly, we searched for nests using a mobile hide intensively within the study site, we recorded the nest location with handheld GPS, and the egg-laying date was estimated based on the floatation stage of each egg in a transparent jar with clean water. Breeding pairs were captured with a walk-in funnel trap placed over the nest, and they were banded with a unique combination of three colour rings and an alpha-numeric metal ring. Nests were monitored every 2–5 days until 20 days of incubation, then were checked every day until hatching to obtain nesting success data. Broods were search intensively daily to determine the date of brood desertion. Re-sightings of previously colour banded plovers were also recorded. Other morphological data (i.e. body weight, tarsus length, beak length) was also collected, however was not used in the analyses.

2.2.2 Data collection

***Quantification of mate fidelity*.** Snowy plovers that were monitored in this study were actively choosing to retain or to divorce their mates. The mating decision of each individual was recorded as either mate retention or divorce in regard to their previous breeding attempt. We evaluated mating decisions separately for banded males and females in the population, since the decisions may influence one another and as such may not be independent. Individuals were included in the analyses if they satisfied the following conditions: (i) we knew the identity of their mate(s), (ii) they were observed in at least two reproductive attempts that were either within or between years, and (iii) if there is a mate change, only those who change their mates while the previous mate is known to be alive are included. In total 149 breeding events (Table 2.1a, 75 divorces in females, 26 divorces in males and 24 retained pairs) fitted the criterion for the within-year mate fidelity analysis from 2006 to 2011. For plovers with more than two nests within a year, only the data from the first two nests were included in the within-year mate fidelity analysis due to the small number of individuals with three or more nests: during the study period, there were only 7 females and 2 males in total that had three breeding attempts.

For individuals with one or multiple nests in each of the two consecutive years, we evaluate between-year mate fidelity in two different ways (see Figure 2.1). First, when an individual's mate

during late season (see "relative egg-laying date" below, late season is when the relative egg-laying date is greater than 0) in year 1, had the same as the mate in early season (when the relative egg-laying date is less than 0) in year 2, it was classified as mate retention, or otherwise divorce (hereinafter Late-early mate fidelity). In total 102 breeding events (Table 2.1b, 42 divorces in females, 38 divorces in males and 11 retained pairs) fitted the criteria for the Late-early mate fidelity. Second, if a plover mated to the same individual in the early seasons of both year 1 and year 2, this was classified as retention, or divorce otherwise (hereinafter Early-early mate fidelity). In total, 116 breeding events (Table 2.1b, 35 divorces in females, 49 divorces in males and 16 retained pairs) fitted the criteria for the Early-early mate fidelity. All individuals were classified into three groups as divorced males, divorced females and retained pairs (Sandercock *et al*. 2000).

Nesting success and reproductive success. Nesting success was quantified based on the fate of the first nest of each individual that were included in our study. The fate of nest was recorded as either successful (at least one chick hatched) or failed (no chicks hatched due to predation, destruction, abandonment, eggs disappeared < 15 days after estimated laying date, eggs did not hatch, or the nest was flooded). We quantified reproductive success as the cumulative number of hatchlings each individual produced in all breeding attempts either within or between years.

Relative egg-laying date. The egg-laying date was used to quantify breeding phenology. We controlled for breeding phenological differences between years by converting egg-laying dates into Julian dates ("lubridate" package in R, Grolemund and Wickham 2011), and calculating the relative egg-laying date using the z-transformation (mean = 0, SD = 1).

Breeding dispersal. Within-year breeding dispersal was defined as the straight-line distance (in meters) between an individual's successive nests within a year. For between-year breeding dispersal, we measured the straight-line distance between (i) the last nest in year 1 and the first nest in year 2, and (ii) the first nests of two consecutive years.

Re-mating time. Re-mating time is defined as the number of days that an individual spent on establishing a new clutch after terminating care of the previous brood. Broods were searched in the breeding area daily. If a parent was missing during two consecutive sightings or seen paired to another plover it was considered to have deserted the brood. We estimated the date of brood desertion for a parent as the mid-point between the time when the individual was last seen with his/her brood and first seen without the brood. We estimated second nest egg-laying date based on the floating stage of the eggs (see above). We only estimated the re-mating times within years.

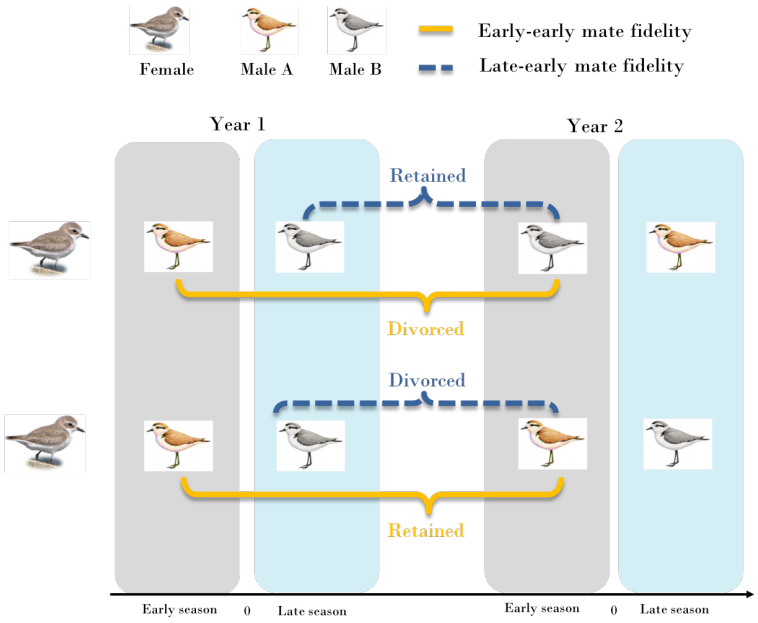

Figure 2.1 Schematic illustration of two estimates of between-year mate fidelity in snowy plovers: Early-late and Early-early mate fidelities.

2.2.3 Statistical analyses

Comparison of male and female mate fidelity. We analysed mating decision as either mate retention or divorce of a plover from an individual its previous breeding attempt. We calculated the number of mate retentions and number of divorces in males and females within the population for both within and between years We used the Two-proportion *z* test (Yau 2013) to compare the proportion of divorced females relative to the female population to the proportion of divorced males relative to the male population both within- and between years.

The relationship between mate fidelity and nesting success. We constructed separate models for males and females to investigate whether mate fidelity is related to nesting success within and between years. Here, separation of the sexes was necessary since nesting success is non-independent variable within a pair, therefore individuals of a pair provide the same data points. In the latter analyses, mate fidelity of an individual was the dependent variable, and nesting success was used as explanatory variable. To analyse the females, we used Generalized Linear Mixed Models (GLMM) with binomial error and included Individual ID and Year as random effect variables to account for the repeated identities of females among years. For males, we used Generalized Linear Model (GLM) with binomial error.

Reproductive success and mate fidelity. To investigate if mate fidelity relates to reproductive success (estimated as the total number of hatchlings from both clutches), we compared divorced

males, divorced females, retained pairs using Kruskal-Wallis tests followed by *post-hoc* pairwise comparisons (Dunn test) adjust by bh to test group differences within and between years.

Breeding dispersal and mate fidelity. Models were built to investigate the relationship between breeding dispersal and mate fidelity groups within and between years. Log-transformed (ln) breeding dispersal was the dependent variable, and mate fidelity groups (divorced males, divorced females and retained pairs) were the explanatory variable. Linear mixed-effects model (LMM) via REML was fitted and maintained Individual ID and Year as random effect variables. Then the estimated marginal means (emmeans from package "emmeans" in R) were calculated for each group, *post-hoc* pairwise comparisons adjusted by Tukey were applied to test group differences.

Re-mating time and mate fidelity. To investigate if re-mating time differs between mate fidelity groups (divorced males, divorced females and retained pairs), we used Kruskal-Wallis tests followed by *post-hoc* pairwise comparisons (Dunn test) to test group differences within and between years. All statistical analyses were performed using R version 3.5.1 (R Core Team 2018).

2.3 Results

2.3.1 Mate fidelity between sexes

Within breeding years, males showed higher mate fidelity than females using 149 breeding events (Table 2.1a, 75 divorces in females, 26 divorces in males and 24 retained pairs) from 2006 to 2011 (Table 2.1a, two-proportion z test, $P = 0.002$, $n = 6$ years). The different numbers of female and male breeding attempts are due to the fact that more females than males had multiple breeding attempts. Between breeding years, however, we did not find a difference in mate fidelity of males vs. females (Table 2.1b, two-proportion z test; Late-early mate fidelity: $P = 1.00$, $n = 5$ years; Early-early mate fidelity: $P = 0.55$, $n = 5$ years).

Table 2.1 Mate fidelity in snowy plover. **(a)** Number of males and females divorced or retained a mate within years, $n = 149$ breeding events. **(b)** Number of males and females divorced or retained a mate between breeding years (Late-early mate fidelity, $n = 102$ breeding events; Early-early mate fidelity, $n = 116$ breeding events; 2006–2011). See Methods for details.

(a) Within years

Year	2006	2007	2008	2009	2010	2011	Total
Number of divorces in females	11	21	10	14	12	7	75
Number of retentions in females	6	8	3	2	3	2	24
Number of divorces in males	5	8	3	3	4	3	26
Number of retentions in males	6	8	3	2	3	2	24

(**b**) **Between years**

Late-early mate fidelity

Year	2006–07	2007–08	2008–09	2009–10	2010–11	Total
Number of divorces in females	12	6	7	12	5	42
Number of retentions in females	4	1	1	3	2	11
Number of divorces in males	8	8	11	7	4	38
Number of retentions in males	4	1	1	3	2	11

Early-early mate fidelity

Year	2006–07	2007–08	2008–09	2009–10	2010–11	Total
Number of divorces in females	13	4	4	7	7	35
Number of retentions in females	1	4	2	6	3	16
Number of divorces in males	17	7	8	7	10	49
Number of retentions in males	1	4	2	6	3	16

2.3.2 Mate fidelity in relation to nesting success and reproductive success

Within breeding years, mate fidelity was related to nesting success since divorce was more likely when the nest hatched successfully, whereas mate retention was more likely if the nest failed (Table 2.2, females: GLMM, $P < 0.001$, male: GLM, $P < 0.001$; Figure 2.2). Between breeding years, however, mate fidelity was not related to nesting success. The latter result was consistent between the Late-early mate fidelity and Early-early mate fidelity (Table 2.2).

Divorced plovers (both males and females) produced significantly more hatchlings within breeding years than those retained their mate. Reproductive success was not different between divorced males and divorced females (Table 2.3, Kruskal-Wallis tests, $P < 0.001$, followed by *post-hoc* pairwise Dunn test; divorced females – retained pairs: *P adjusted* < 0.001, divorced males – retained pairs: *P adjusted* = 0.05, divorced females – divorced males: *P adjusted* = 0.07; Figure 2.3). Between breeding years, however, reproductive success was not different between divorced and retained individuals neither in the Late-early nor in the Early-early comparisons (Kruskal-Wallis tests; Late-early mate fidelity: $\chi^2 = 0.20$, df = 2, $P = 0.90$; Early-early mate fidelity: $\chi^2 = 4.21$, df = 2, $P = 0.12$).

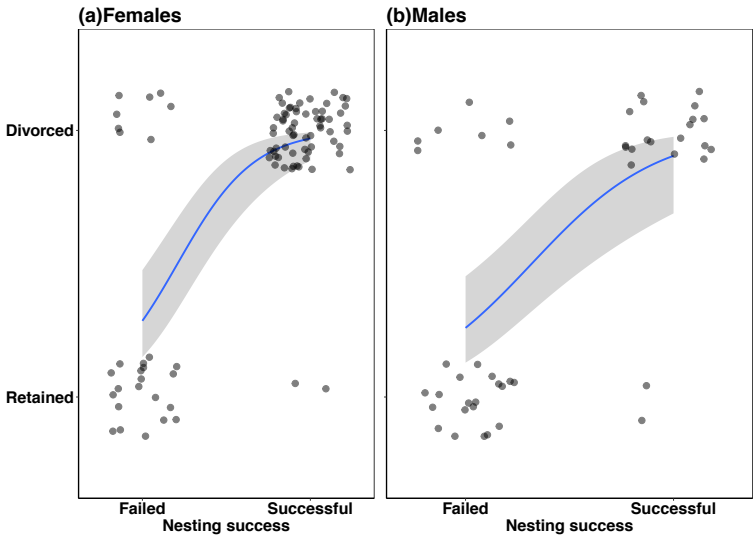

Figure 2.2 Mate fidelity in relation to nesting success in (a) female and (b) male snowy plovers within a year (see Table 2.2 for statistics). Logistic linear regression lines (blue) with standard error (grey).

Table 2.2 Mate fidelity in relation to nesting success within and between breeding years in snowy plover. Generalized Linear Mixed Models (GLMM) with *binomial* error family and including "Individual ID" and "Year" as random effect variables to account for the repeated identities of female individuals among years. For males, Generalized Linear Model (GLM) with *binomial* error family was used. SE = standard error.

Response variable	**Model used**	**Explanatory variable**	**Estimate**	**SE**	***z* value**	***P* value**
Within years						
Female						
Mate fidelity	*Binomial* (GLMM)	Intercept	-0.92	0.42	-2.19	**0.03**
		Nesting success	4.38	0.83	5.27	**<0.001**
Male						
Mate fidelity	*Binomial* (GLMM)	Intercept	-15.92	5.16	-3.09	**0.002**
		Nesting success	29.17	7.59	3.84	**<0.001**
Between years: Late-early mate fidelity						
Female						
Mate fidelity	*Binomial* (GLMM)	Intercept	1.39	0.79	1.75	0.08
		Nesting success	-0.06	0.87	-0.07	0.95
Male						
Mate fidelity		Intercept	1.50	0.78	1.92	0.05

	Binomial (GLM)	Nesting success	-0.33	0.87	-0.38	0.70
Between years: Early-early mate fidelity						
Female						
Mate fidelity	*Binomial* (GLMM)	Intercept	1.47	1.19	1.23	0.22
		Nesting success	-0.75	1.25	-0.60	0.55
Male						
Mate fidelity	*Binomial* (GLM)	Intercept	2.30	1.05	2.20	**0.03**
		Nesting success	-1.37	1.10	-1.26	0.21

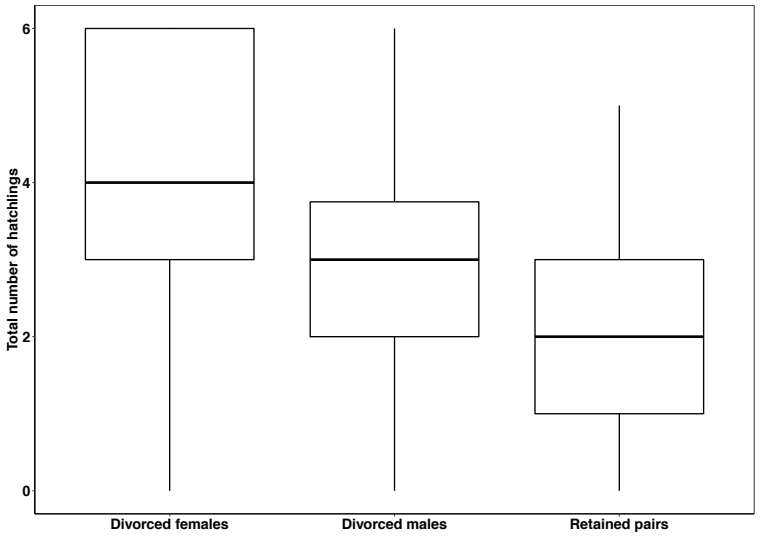

Figure 2.3 Reproductive success in relation to divorce or mate fidelity in snowy plovers (see Table 2.3 for statistics). Medians, upper and lower quartiles, as well as extreme values are shown.

Table 2.3 Comparison of reproductive success between mate fidelity groups (divorced males, divorced females and retained pairs) within breeding years (Kruskal-Wallis tests, $P < 0.001$, followed by *post-hoc* pairwise Dunn test adjust by bh).

Groups	***Z***	***P unadjusted***	***P adjusted***
Within years			
Divorced females – divorced males	1.97	<0.001	0.07
Divorced females – retained pairs	4.08	<0.001	**<0.001**
Divorced males – retained pairs	1.92	<0.001	**0.05**

2.3.3 Mate fidelity in relation to breeding dispersal and re-mating time

Divorced females bred further away than divorced males both within and between years (Figure 2.4, Table 2.4). Divorced males, however, did not breed further away than retained pairs (Table 2.4). Finally, re-mating times were not different between divorced males, divorced females and retained pairs (Kruskal-Wallis test, $\chi^2 = 2.00$, df = 2, $P = 0.37$).

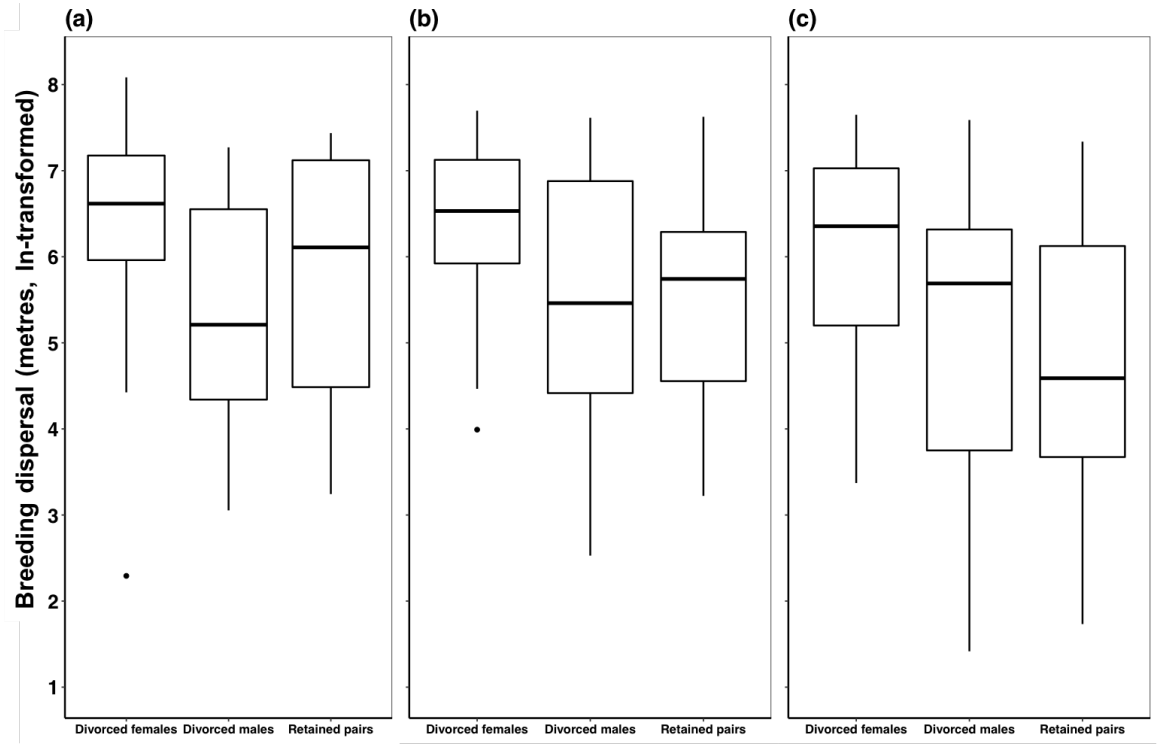

Figure 2.4 Breeding dispersal (a) within-year, and between year (b, Late-early) and (c, Early-early) in snowy plover (see Methods for explanations and Table 2.4 for statistics). Breeding dispersal was estimated in meters and log-transformed (ln). Medians, upper and lower quartiles, as well as extreme values are shown.

Table 2.4 (a) Breeding dispersal in relation to mate fidelity groups (divorced males, divorced females and retained pairs) within and between breeding years. The Linear mixed-effects model (LMM) via REML was fitted and maintained "Individual ID" and "Year" as random effect variables. SE = standard error.

Response variable	Model used	Explanatory variable	Estimate	SE	*t* value
Within years					
Breeding dispersal	LMM	Intercept	6.46	0.16	38.85
		Divorced males	-0.95	0.26	-3.63
		Retained pairs	-0.67	0.26	-2.58
Between years: Late-early					
		Intercept	6.41	0.21	30.25

Breeding dispersal	LMM	Divorced males	-1.01	0.30	-3.37
		Retained pairs	-0.70	0.39	-1.77
Between years: Early-early					
Breeding dispersal	LMM	Intercept	5.87	0.29	20.48
		Divorced males	-0.95	0.38	-2.53
		Retained pairs	-0.73	0.33	-2.23

(b) Comparison of breeding dispersal between mate fidelity groups (divorced males, divorced females and retained pairs) within and between breeding years.

Groups	**Estimate**	**SE**	**df**	***t* ratio**	***P* value**
Within years					
Divorced females – divorced males	0.95	0.26	111	3.60	**0.001**
Divorced females – retained pairs	0.67	0.26	112	2.57	**0.03**
Divorced males – retained pairs	-0.28	0.26	68	-1.05	0.55
Between years: Late-early					
Divorced females – divorced males	1.01	0.31	72	3.28	**0.005**
Divorced females – retained pairs	0.70	0.41	72	1.70	0.21
Divorced males – retained pairs	-0.32	0.44	85	-0.71	0.76
Between years: Early-early					
Divorced females – divorced males	0.95	0.38	70	2.51	**0.04**
Divorced females – retained pairs	0.73	0.35	56	2.09	0.10
Divorced males – retained pairs	-0.22	0.44	88	-0.50	0.87

2.4 Discussion

Previous analyses of mate fidelity were typically concerned with either within-year or between-year mate fidelity and focus largely on monogamous systems (sometimes termed mate desertion, mate abandonment or mate change; Black 2001, Bried *et al*. 2003, Flodin and Blomqvist 2012) Here we take an integrative approach and investigate mate fidelity both within and between breeding years. Using a sequential polygamous shorebird, the snowy plover, we identified factors that predict mate fidelity and its spatial-temporal manifestation in a system, in which males and females differ in their breeding strategies and reproductive efforts.

Our analyses revealed three major results. First, males exhibit higher within-year mate fidelity than females. This is consistent with the previous studies of snowy plover since females tend to desert the brood whereas males are usually the ones that rear the young (Warriner *et al*. 1986, Carmona-Isunza *et al*. 2015). We suggest that male-biased adult sex ratio entices female parents more than male parents to desert their brood and breed again (Stenzel *et al*. 2011, Eberhart-

Phillips *et al*. 2017). thereby resulting in different re-mating opportunities and mate fidelities between males and females. The latter results are consistent with experimental and empirical studies that show altered adult sex ratios influences mating decisions (Karlsson *et al*. 2010, Silva *et al*. 2010, Liker *et al*. 2013, 2014).

However, between years both male and female snowy plovers demonstrated low mate fidelity. We note however that our mate fidelity (and consequently, our divorce decision as well) was based on local returning rates: if paired birds may breed outside the study area and/or some of the survived adults may not return to breed to Ceuta, these survival estimates can be biased. The annual return rates to Ceuta are 41.5% for males (n = 378 individuals) and 35.4% for females (n = 339 individuals, 2006-2011). Therefore, further investigation is required to estimate more precisely the return rates using more comprehensive spatial coverage by visiting additional breeding sites near Ceuta and/or using GPS tags to monitor the movements of adults within- and between years.

Second, divorce was more likely after a nest hatched than after it failed since failed breeders typically re-nested with the same partner. Therefore, divorced plovers, counterintuitively, reared more offspring than faithful individuals. This finding is not consistent with studies of long-lived bird species where low breeding success may trigger divorce (Coulson 1966, Bried *et al*. 2003). We propose that by abandoning the brood and divorcing, individuals try to maximize their reproductive success by producing as many clutches over the season as possible. Divorce may be facilitated by two aspects of natural history: first, nest and chick mortality in this population tend to be high and stochastic, thus parents may need several trials to produce at least some fledglings (Cruz-López *et al*. 2017). Second, the chicks are precocial and thus they only require modest care: brooding and protection, but not feeding (Székely *et al*. 1999). The well-developed hatchling then gives the opportunity for one parent to terminate care and start breeding with a new partner (Székely *et al*. 1996, McNamara *et al*. 1999, Houston *et al*. 2013). Mate retention was, however, more likely after nest failure, in which case the parental duties of both parents terminated at the same time, therefore, the fastest way to breed again was to retain the previous partner ("fast-track hypothesis"; Zann 1994, Fowler 1995, Perfito *et al*. 2007).

However, breeding success in previous years may have little impact on the re-mating decision of snowy plovers. We presume that the breeding time constraint facilitates early breeding with available mates instead of waiting for the former partner, especially since early breeding is associated with higher nest survival (Székely *et al*. 1999, van de Pol *et al*. 2006, Plaschke *et al*. 2019). Since snowy plovers only have about two years of breeding life (average breeding life of males: 2.3 ± 1.6 years; females: 1.9 ± 1.2 years; Colwell *et al*. 2013), they may not discriminate against previous mates even if they were failed breeders Furthermore, returning to the breeding ground may be stochastic and this can also produce decoupling between nesting success and mate fidelity (Handel and Gill 2000, Bried *et al*. 2003, Gilsenan *et al*. 2017).

Third, we found that females tend to disperse farther than males after divorce both within and between breeding years. This follows the general pattern of female-biased breeding dispersal observed in most bird species including shorebirds (Greenwood and Harvey 1982, Clarke *et al*. 1997, Sandercock *et al*. 2000, Liu and Zhang 2008). However, in polyandrous birds like snowy plovers there is an additional reason: finding new mate whilst their previous mate is taking care of the chicks (D'Urban Jackson *et al*. 2017). For males, returning to previous breeding site – that is often thought as a high-quality site providing good brood rearing opportunities (Sandercock *et al*. 2000) – is a factor that reduces their aptitude for moving large distance between nests. Mate fidelity is often related to the degree of site fidelity (Cezilly and Johnson 1995, Cezilly *et al*. 2000), and whilst it would be tempting to argue that higher mate fidelity leads to higher site fidelity in males, or vice versa high divorce rate by females lead to more extensive breeding dispersal, to conclude the directionality of causation – and to separate whether the males or the females drive these relationships – would require experimental manipulation of mate fidelity, site fidelity or both.

Together, our results support theoretical arguments that divorce is an adaptive strategy by which individuals improve their reproductive success (Black 1996, Dubois and Cézilly 2002). Divorced birds reached higher number of breeding attempts and higher breeding success than individuals that retained their mates, at least within years. We suggest that in snowy plovers, divorce is result from their effort to maximise reproductive output during a given time period. The birds' urge to re-mate as many times as possible within a breeding season and produce the highest possible number of chicks could be traded off by lowered survival of their abandoned broods although given the precocity of the young, this cost may not be prohibitive (Székely and Williams 1995). We suggest that the urge for a fast reproduction in snowy plovers is an adaptive response to life histories (i.e. short life span) and breeding parameters (i.e. short breeding period and breeding success). Additionally, time constraint in breeding confounded with the bias in population demography (i.e. male-biased adult sex ratio) propels both sexes adopt different mating strategies, resulting in different spatial dispersal patterns. Therefore, mate choice and breeding dispersal are important components of their breeding strategy. We encourage further investigations of breeding strategy including mate fidelity between different polygamous shorebird populations, and to understand the generality of our findings across the various natural populations with the intention of informing conservation decisions.

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Ethics statement

All aspects of the fieldwork were authorized by the national authorities of Mexico (Secretaría de Medio Ambiente y Recursos Naturales, SEMARNAT; SGPA/DGVS/01717/10, SGPA/DGVS/01367/11). Birds were ringed and handled by trained people aiming to cause as little disturbance to birds as possible.

Conflict of interest

Authors have no conflict of interest to declare.

Data accessibility

Data are available from the Dryad Digital Repository, doi:10.5061/dryad.3185h66

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Chapter 3 | Successful breeding predicts divorce in plovers

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Authors' Contributions

NH and TS conceived the project and designed methodology;
KK, MCCI, DBR, DC, JJHSC, JC, JF, MY, MJ, MM, MCL, MS, MAW, PL, PL, PQ, TM, UB, YL, AK provided the data;
NH, JOV, VK carried out the statistical analyses.
NH, TS and GCM led the writing of the manuscript.
All authors contributed critically to the drafts.

Abstract

When individuals breed more than once, parents are faced with the choice of whether to re-mate with their old partner or divorce and select a new mate. Evolutionary theory predicts that, following successful reproduction with a given partner, that partner should be retained for future reproduction. However, recent work in a polygamous bird, has instead indicated that successful parents divorced more often than failed breeders, because one parent can benefit by mating with a new partner and reproducing shortly after divorce. Here we investigate whether successful breeding predicts divorce using data from 14 well-monitored populations of plovers (*Charadrius* spp.). We show that successful nesting leads to divorce, whereas nest failure leads to retention of the mate for follow-up breeding. Plovers that divorced their partners and simultaneously deserted their broods produced more offspring within a season than parents that retained their mate. Our work provides a counterpoint to theoretical expectations that divorce is triggered by low reproductive success, and supports adaptive explanations of divorce as a strategy to improve individual reproductive success. In addition, we show that temperature may modulate these costs and benefits, and contribute to dynamic variation in patterns of divorce across plover breeding systems.

Keywords

Mating system, divorce, pair-bond, parental care, breeding dispersal, breeding success, shorebirds, adult sex ratio

3.1 Introduction

The decision to retain or divorce a mate between successive breeding events is an important aspect of mating systems (Reynolds 1996), with direct implications for reproductive success and subsequent survival of the parents (Neff and Pitcher 2005, Székely *et al*. 2006, Culina *et al*. 2013, Culina *et al*. 2014, Székely *et al*. 2014). Mate fidelity, defined as retaining the same mate for subsequent breeding attempt(s), is commonly observed in a variety of taxa (Møller 2003, Lukas and Clutton-Brock 2013). Mate fidelity varies widely among species in terms of duration, with some exhibiting short-term mate fidelity within a single season, in which an individual remains faithful to a mate throughout one breeding season and initiates another breeding season with a new mate while the old partner is still alive, whereas other species show long-term (i.e. between seasons) or even life-time mate fidelity (Black 1996, Black 2001, Møller 2003, Reichard and Boesch 2003, Lukas and Clutton-Brock 2013, Sanchez-Macouzet *et al*. 2014). Understanding the drivers of interspecific variation in mate fidelity is thus crucial to understand the evolutionary diversity of animal mating systems.

Various factors have been proposed to explain variation in mate fidelity across taxa. The abiotic environment (such as temperature and precipitation) often shapes mating decisions. Variation the in abiotic environment may affect resource availability and the duration of suitable breeding periods, creating different ecological constraints that may limit or promote mate fidelity (Blomqvist *et al*. 2001, Botero and Rubenstein 2012, Eberhart-Phillips 2019). For example, arctic bird species have typically short breeding seasons due to the harsh and stochastic environmental conditions, and tend to exhibit high fidelity to a mate, which is likely to improve offspring survival (Green *et al*. 1977, Saalfeld and Lanctot 2015). In contrast, mild environments in temperate and tropical regions tend to provide a more prolonged breeding season so that an individual might initiate multiple clutches with the same or different mates (Székely *et al*. 1999, Yasue and Dearden 2008, Halimubieke *et al*. 2019). The influence of environmental conditions on mating decisions has been observed in a variety of taxa including flies, fish, frogs and birds (Gilburn and Day 1994, Welch 2003, Candolin *et al*. 2007). Aspects of the social environment, such as adult sex ratio (ASR) are also known to influence mating decisions (Lode *et al*. 2004). In species or populations with a biased ASR, the rare sex is more likely to initiate divorce since the rare sex has higher mate availability than the common sex (e.g. frogs and birds; Lode *et al*. 2004, Liker *et al*. 2014, Parra *et al*. 2014). Life history traits may also influence rates of mate fidelity; for instance, species with a high divorce rate have a high mortality rate, whereas species with high adult survival rate (or long-lived species) tend to retain the same mates from year to year (Bried *et al*. 2003, Jeschke and Kokko 2008), suggesting that survival rate or longevity predict mating decisions. Although life history theory predicts that large body size is usually related to high survival rate and longevity (Black 1996), there is sparse evidence to show whether larger species exhibit stronger mate fidelity than smaller ones (Black 1996, Bried *et al*. 2003). Sexual size dimorphism (SSD) may also relate to

patterns of mate fidelity, since more exaggerated SSD may reflect more intense sexual selection and polygamy (Andersson 1994). Other life history traits (e.g. age of first reproduction, life span) have also been proposed to be linked to mate fidelity (Choudhury 1995, Wheelwright and Teplitsky 2017).

Several hypotheses have been put forward to explain variation in mate fidelity, emphasizing the costs and benefits of individual mating decisions, and their relationship with breeding success or breeding time. The "*fast-track hypothesis*" suggests that individuals retain a mate to reduce the time and energy costs of searching for a new mate (Adkins-Regan and Tomaszycki 2007, Perfito *et al*. 2007). The "*mate familiarity hypothesis*" highlights that retaining a mate could improve breeding performance by enhancing coordination between parents and thereby improving reproductive success (Ens *et al*. 1996, Gabriel and Black 2013, Sanchez-Macouzet *et al*. 2014). In contrast, changing a mate may be beneficial in some long-lived species, as individuals may divorce their current partner to mate with relatively higher quality or more compatible partners in order to improve breeding success ("incompatibility hypothesis"; Coulson 1966, Kempenaers *et al*. 1998); as a corollary, successful breeding pairs are more likely to stay together for future breeding attempts (Black 2001, Pyle *et al*. 2001, Flodin and Blomqvist 2012). It has also been suggested that divorcing and rapidly changing a mate may be favoured by some species in order to make the most out of a restricted time budget (e.g. short life span or short breeding season; Arnqvist and Nilsson 2000, Halimubieke *et al*. 2019).

Mating decisions are also associated with breeding dispersal, i.e. the movement of an adult from one breeding location to another between consecutive breeding attempts within a breeding year (Greenwood 1980, Clobert *et al*. 2001). Breeding dispersal may exhibit sex differences as males and females can adopt different mating strategies, for instance, the more polygamous sex is more likely to disperse farther to find new mating partners than the less polygamous sex (Greenwood 1980, Trochet *et al*. 2016, D'Urban Jackson *et al*. 2017, Székely 2019). Studies also suggest that mate fidelity can be a by-product of site fidelity (Morse and Kress 1984, Bried *et al*. 2003), and conversely, mate change can be a result of changing nest sites (Pietz and Parmelee 1994, Thibault 1994). Nonetheless, studies of mate fidelity mostly centre around socially monogamous bird species between years (Dubois and Cezilly 2002, Bried *et al*. 2003), and studies that investigate mate fidelity in multiple species and populations that exhibit variable duration of pair-bonds within a single breeding season are scarce (Halimubieke *et al*. 2019).

Here we focus on *Charadrius* plovers – small ground-nesting shorebirds – for four reasons. First, they exhibit intra- and interspecific variation in several behavioural, ecological, demographic and life history traits (Eberhart-Phillips 2019, Székely 2019), making them an excellent model system for addressing mate choice decisions. Second, plovers are globally distributed, breeding on all continents except Antarctica, providing an excellent opportunity to conduct a geographically large-scale study (Eberhart-Phillips 2019, Székely 2019). Third, they display flexible mating

systems including short-term within-year pair-bonds. In some plover populations, both males and females may have up to four breeding attempts with the same or different mate sequentially within a single season (Kosztolányi *et al*. 2006, del Hoyo *et al*. 2018, Székely 2019). Finally, their breeding biology is well characterised: plovers typically lay two to four eggs (depending on the species) in poorly insulated nest scrapes with both parents typically providing care during the incubation stage (del Hoyo *et al*. 2018). Plover chicks are precocial and nidifugous, and although in most species post-hatch care is provided by both parents, in others, either parent (usually females) may desert their mate during brood care to become polygamous (Kosztolányi *et al*. 2006, del Hoyo *et al*. 2018). Furthermore, plovers show low extra-pair paternity rates (less than 5%), indicating that social mates are a good proxy for genetic mates and thus the reproductive success of social pairs accurately reflects Darwinian fitness (Maher *et al*. 2017).

In a recent study, Halimubieke *et al.* (2019) reported that snowy plovers (*Charadrius nivosus*), especially females, are more likely to divorce after successful nesting, simultaneously deserting their current brood, and initiate a new breeding attempt with a different mate, whereas pairs tend to stay together after failed breeding attempts and initiate a second nesting attempt with the same mate. Divorcing individuals reared more offspring than those that retained their mates. This difference in mating strategy between male and female snowy plovers led to female-biased breeding dispersal, as females divorced their mates more often than males and subsequently dispersed to pursue additional mating opportunities.

Here we use data from 8 plover species across 14 populations (see Table 3.1 and Figure 3.1 for study sites, species and study periods) to investigate four questions. First, we explore the variation in mate fidelity in both males and females across populations within breeding years, and the abiotic environmental and life history correlates of variation in mate fidelity across populations. We characterise the mean ambient temperature and temperature variation (see Methods for details), and expect that colder ambient temperature and greater temperature variation promote mate fidelity. We also investigate body weights and the sexual size dimorphism (SSD, see Methods) as life history traits, and expect that populations with heavier plovers (or less extensive SSD) show higher mate fidelity rate than populations with small plovers and more extensive SSD – based on studies that show sexual selection is associated with the extent of SSD (Székely 2004, Székely *et al*. 2007). Second, we evaluate the generality of the previous study in snowy plover (Halimubieke *et al*. 2019), and expect that successful breeding leads to divorce whereas failed breeding leads to mate fidelity. Third, we investigate the fitness consequences of mating decisions, and expect that birds that divorce and desert their broods have higher reproductive success than individuals that retained their mates within breeding seasons. Finally, we investigate whether mating decisions are related to breeding dispersal, as we expect that individuals that divorce their previous mate disperse greater distances to initiate another breeding attempt with a different mate than those who retain their existing mate for their next breeding attempt (Halimubieke *et al*. 2019).

3.2 Methods

3.2.1 Study site and fieldwork

Fieldwork was carried out in 14 breeding populations of 8 plover species and ranged from 1 to 9 breeding seasons per population (see Table 3.1 and Figure 3.1 for study sites and study periods). Egg-laying date of nests was either known (for nests that were found during egg-laying) or estimated by floating eggs or measuring egg mass relative to egg size (Székely *et al*. 2008, Parra *et al*. 2014). Breeding pairs were captured on their nest while incubating eggs, using funnel traps, noose mats, box traps or bownet traps. Morphological data of each individual were collected: body weight was collected from eight populations (body weight data from six populations were not collected in the field, see below for details); sex was determined by morphological features, for monomorphic species, molecular sexing was applied to identify the sex of the individual. Finally, each individual was banded with a unique combination of colour rings/flags and a metal ring (see Appendix S2 in Supplementary Material and further references in Székely *et al.* (2008). Nests were monitored until hatching to obtain nesting success data (see below for details).

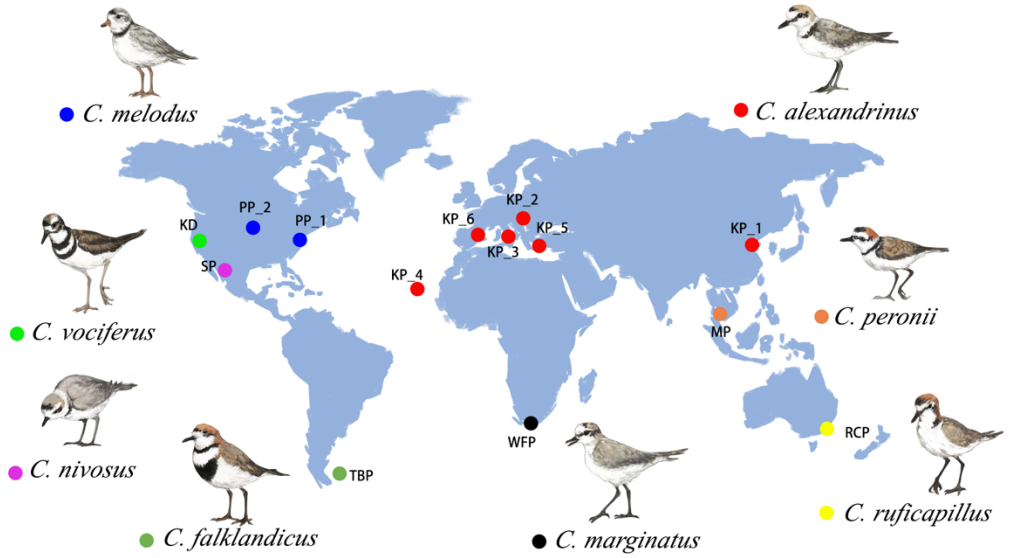

Figure 3.1 Study sites and plover populations used in this study (n = 14 populations, 8 species). Original plover illustrations and map by Siyu Ding.

3.2.2 Data collection

Quantification of mate fidelity and mate fidelity rate within breeding years. Plovers that were included in this study could freely retain or divorce their mates in natural conditions without manipulation. The mating decision of each individual was recorded as either *mate retention* or *divorce* with respect to their previous breeding attempt within each breeding year. The mating decision of males and females were not independent from each other, therefore, we assessed mating

decisions separately for banded males and females in each population. We used the same criteria, following Halimubieke *et al.* (2019), for individuals that were included in the analyses: (a) the identities of the individuals and their mate(s) were known, (b) they were observed in at least two reproductive attempts within a breeding year, and (c) if there was a mate change, only those who changed their mates while the previous mate was known to be alive were included. In total, 1927 breeding events (Table 3.1, 124 divorces in males, 205 divorces in females, and 799 retentions in both sex) fitted the criteria for the mate fidelity analysis in the 14 populations. Mate fidelity rate represents the proportion of retained individuals in given breeding year(s) in each population.

Abiotic environment, body weight and sexual size dimorphism (SSD). In this study, the ambient temperature and temperature variation of each population over the study period are used as the proxies of abiotic environmental conditions. We extracted high resolution historical daily temperature data collected by the nearest weather stations for each study site from the National Oceanic and Atmospheric Administration (NOAA) database and University of East Anglia Climate Research Unit database (CRU; http:// www.cru.uea.ac.uk/, version 3.10.01), using the R package "rnoaa" (Chamberlain *et al*. 2017). The average distance between weather stations and study sites is 60.17 km (see Table S2 for more details). If the weather record was incomplete for any study site, we used the R package "GSODR" (Sparks *et al*. 2017) to extract weather data from the USA National Center for Environmental Information (NCEI) database. Since our study focused on breeding behaviour, we only extracted daily temperatures from each month of the breeding season(s) when capture data were collected in a given population. and we calculated the average temperature of the breeding season(s); we refer to this variable as ambient temperature. The temperature variation refers to average between-year fluctuations in ambient temperature, and it was calculated as the standard deviation of the average temperature of all breeding years for a given population.

The average body weight of birds from each population was calculated from individual body weight data collected from the fieldwork over the study period. We also searched for average body weight data in Handbook of the Bird of the World (del Hoyo *et al*. 2018) and CRC Handbook of Avian Body Masses (Dunning 2007) for the following populations for which we did not have field data: the Kentish plover population from Italy, two piping plover populations from USA, the red-capped plover population, the white-fronted plover population and the Malaysian plover population. To quantify sexual size dimorphism, we divided the male average body weight by that of the female and log-transformed this ratio, and assigned positive signs when males were the larger sex and negative ones when females were larger.

Nesting success and egg-laying date. Nesting success was determined based on the fate of the nest(s) of each individual included in our study. The fate of a nest was recorded as either successful (at least one chick hatched) or failed (no chicks hatched due to predation, destruction, abandonment,

eggs disappeared <15 days after estimated laying date, eggs did not hatch, or the nest was flooded). The nesting success rate represents the proportion of nests with at least one successfully hatched egg in each population over the study period. The egg-laying date was used to quantify breeding phenology. We controlled for breeding phenological differences between years by converting egg-laying dates into Julian dates ("lubridate" package in R; Grolemond and Wickman 2011), and standardised egg-laying date using the z-transformation (mean = 0, SD = 1).

Reproductive success and breeding dispersal. Reproductive success was quantified as the cumulative number of hatchlings each individual produced in all breeding attempts within each breeding year. We did not use the number of fledglings as the proxy for reproductive success as the fates of fledglings are difficult to estimate in precocial species like plovers due to the high mobility and camouflage of broods (Eberhart-Phillips 2019). Breeding dispersal was defined as the straight-line distance (in meters) between an individual's successive nests within a year for those populations with nest location data.

3.2.3 Statistical analyses.

To investigate variation in mate fidelity rate across populations, first, we used analysis of variance ANOVA to compare the mate fidelity rates of both sexes across 14 populations. We then constructed two generalised linear mixed models (GLMM) via Template Model Builder (TMB) with binomial error structure to test environmental and life history predictors of mate fidelity rate in both sexes. In these models, the mate fidelity rate (male or female) of each population over the study period was the dependent variable. Ambient temperature, temperature variation and nesting success rate of each population over the study period, alongside average body weight (male or female) and SSD of the populations were used as explanatory variables. Species and population were included as random effects.

To investigate individual mating decisions, we first constructed a GLMM for each sex with a binomial error structure, and examined how mate fidelity relates to nesting success and relative egg-laying date. A similar model for each sex was developed to explore mate fidelity in relation to daily temperature and individual body size. In these models, species, population, individual identity and year were used as random effect variables.

Next, we used a GLMM to investigate if reproductive success is related to mate fidelity by comparing the total number of hatchlings from all clutches among divorced males, divorced females and retained pairs. A Poisson error structure was used because: (i) Gaussian version of the model suggested normality assumptions were violated; (ii) reproductive success is a count and thus an integer variable. Species, population and individual identity were used as random effects.

To investigate the relationship between breeding dispersal and mate fidelity, we use same methods as Halimubieke *et al*. (2019) We built a linear mixed-effects model (LMM) using log-

transformed (ln) breeding dispersal as the dependent variable, and mate fidelity groups (divorced males, divorced females and retained pairs) as the explanatory variable. LMM via REML was fitted and included population and individual identity as random effect variables. The goodness-of-fit test showed that the residuals of the model show equal variances and follow normal distribution, supporting the validation of the model we used.

Estimated marginal means (emmeans from package "emmeans" in R; Searle *et al*. 1980) were calculated for each group in the latter two models, and *post-hoc* pairwise comparisons adjusted by Tukey were applied to test group differences. There was no model simplification, and all terms were retained in all the models above.

To test whether phylogenetic relatedness influenced our results, we followed the same method as Vincze *et al.* (2017), the above models were repeated using Bayesian Markov chain Monte Carlo GLMM (MCMCglmm; Hadfield 2010), including a correlational structure based on the species-level phylogenetic tree of the 8 *Charadrius* species studied here. The phylogenetic signal of the investigated trait in these models was low (model description and calculation of the phylogenetic signal are given in Appendix S1). All statistical analyses were performed using R version 3.5.1 (R Core Team 2018).

Table 3.1 List of plovers *Charadrius* spp. populations used in the study (8 species, 14 populations). See Appendix S2 for references for populations used in this study.

Species	English name	Population	Code	Coordinates	Years of data collection	No. divorced male	No. divorced female	No. Retention
C. alexandrinus	Kentish plover	Bohai Bay (China)	KP_1	39º09'N, 118º09'E	2016-2018	7	5	3
C. alexandrinus	Kentish plover	Great Hungarian Plain (Hungary)	KP _2	46º40'N, 19º10'E	1989-1994	4	7	16
C. alexandrinus	Kentish plover	Senigallia (Italy)	KP _3	43°43'N, 13°12'E	2011-2017	14	12	21
C. alexandrinus	Kentish plover	Maio Island (Cape Verde)	KP _4	15º09'N, 23º13'W	2009-2010; 2013-2015	3	1	13
C. alexandrinus	Kentish plover	Tuzla Lake (Turkey)	KP _5	36º42'N, 35º03'E	1996-1999	23	26	29
C. alexandrinus	Kentish plover	Llobregat Delta (Spain)	KP _6	41º18'N, 02º08'E	2004-2007	5	5	9
C. melodus	piping plover	New Jersey (USA)	PP_1	39°04'N, 74°43'W	2012-2013; 2015-2016	3	6	48
C. melodus	piping plover	Great Plain (USA)	PP_2	42°51′N, 97°29′W	2005-2009; 2012-2015	17	45	44

C. ruficapillus	red-capped plover	Altona (Cheetham) Saltworks (Australia)	RCP	37º53'S, 144º47'E	2010-2013	1	0	16
C. nivosus	snowy plover	Ceuta Bay (Mexico)[8]	SP	23º54'N, 106º57'W	2006-2012	26	79	29
C. falklandicus	two-banded plover	Sea Lion Island (Falklands)[9]	TBP	51º41'S, 59º10'W	2006-2008	2	6	17
C. vociferus	killdeer	Honey Lake, California (USA)[10]	KD	40°7'N, 120°14'W	1993-1997; 99-2001	18	11	106
C. marginatus	white-fronted plover	Cape Peninsula (South Africa)	WFP	34º08'S, 18º20'E	1999-2000; 2002-2004; 2006	0	0	439
C. peronii	Malaysian plover	Prachuap Khiri Khan (Thailand)[11-14]	MP	12º00'N, 99º53'E	2004-2005	1	2	9

3.3 Results

3.3.1 Mate fidelity rate

Mate fidelity rates (proportion of retained individuals in given breeding season; see Methods for further details) of both males and females were different between plover populations in both sexes (see Figure 3.2; male: $F = 8.33$, $df = 13$, $P < 0.001$; female: $F = 6.34$, $df = 13$, $P < 0.001$; one-way ANOVA).

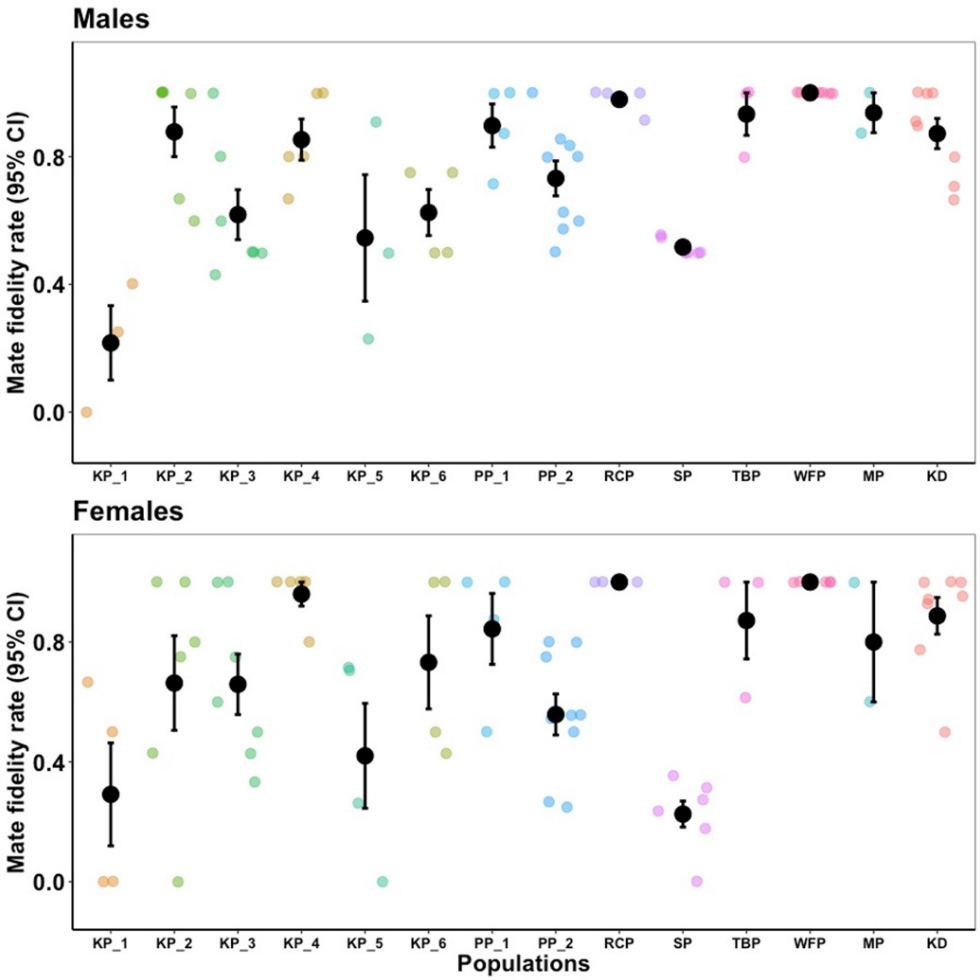

Figure 3.2 Mate fidelity rates in plover populations. Annual mate fidelity rate of each population is shown in different colours. Means of annual mate fidelity rate of each population, lower and upper 95% confidence intervals are shown. See Table 3.1 for details of the population codes.

3.3.2 Environmental and life history predictors of mate fidelity

Mate fidelity rate decreased with ambient temperature (i.e., mean temperature over breeding season, see Methods; Table 3.2). Males (but not females) in warmer climates had lower mate fidelity than individuals that breed in colder environments (Figure 3.3a). However, mate fidelity was unrelated to temperature variation (i.e., between-year fluctuations in ambient temperature, see Methods). Within populations, mate fidelity was unrelated to daily temperature in both males ($P = 0.41$, $N =$

788 observations) and females (P = 0.69, N = 776 observations; see Table S1 in the Supporting information).

Mate fidelity rates were unrelated to body weight or SSD between populations (Table 3.2; see Methods), nor within populations (male: P = 0.34, N = 136 observations; female: P = 0.12, N = 193 observations; GLMM; Table S1).

3.3.3 Mate fidelity in relation to nesting success and egg-laying date

Mate fidelity rate declined with nesting success rate (i.e., proportion of successfully hatched nests) since populations with high nesting success rates showed lower mate fidelity rate (Table 3.2, Figure 3.3b). Consistently, within populations mate fidelity was related to nesting success, as divorce was more likely when the nest hatched successfully, whereas mate retention was more likely if the nest failed (Figure 3.4; Table 3.3). However, egg-laying date was not significantly related to mate fidelity (Table 3.3).

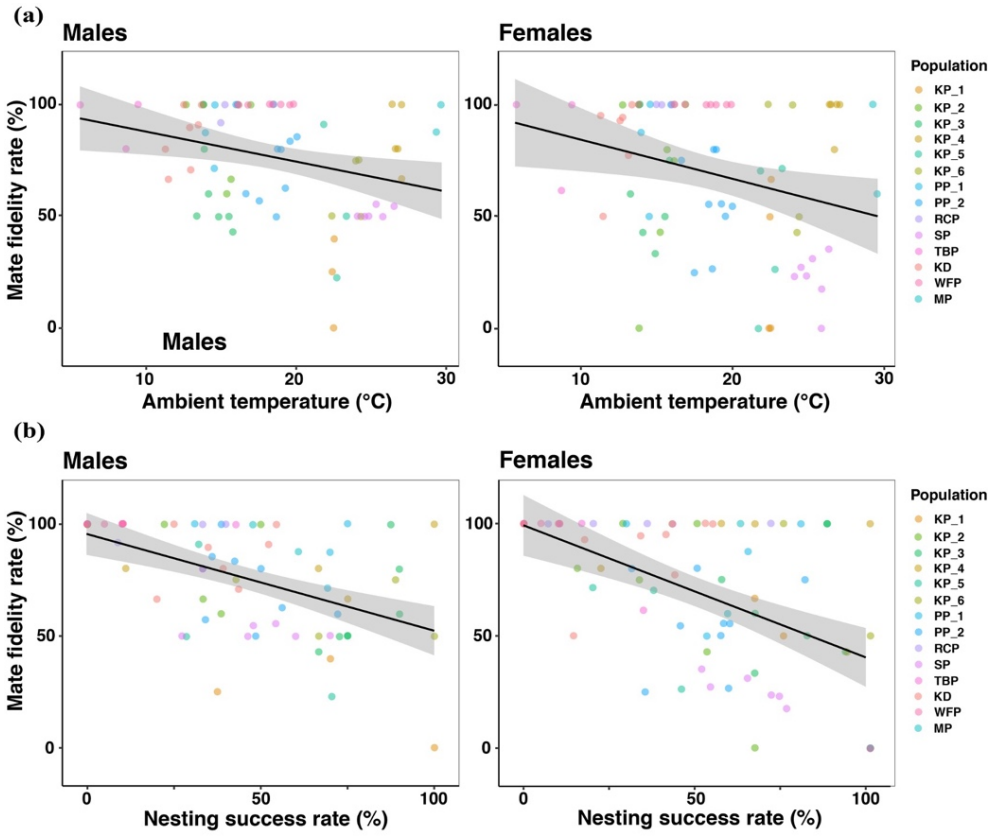

Figure 3.3 Mate fidelity rate in relation to **(a)** ambient temperature and **(b)** nesting success rate in male and female plovers (see Table 3.2). Annual mate fidelity rate of each population is shown in different colours. Linear regression lines are shown in black with lower and upper 95% confidence intervals (grey).

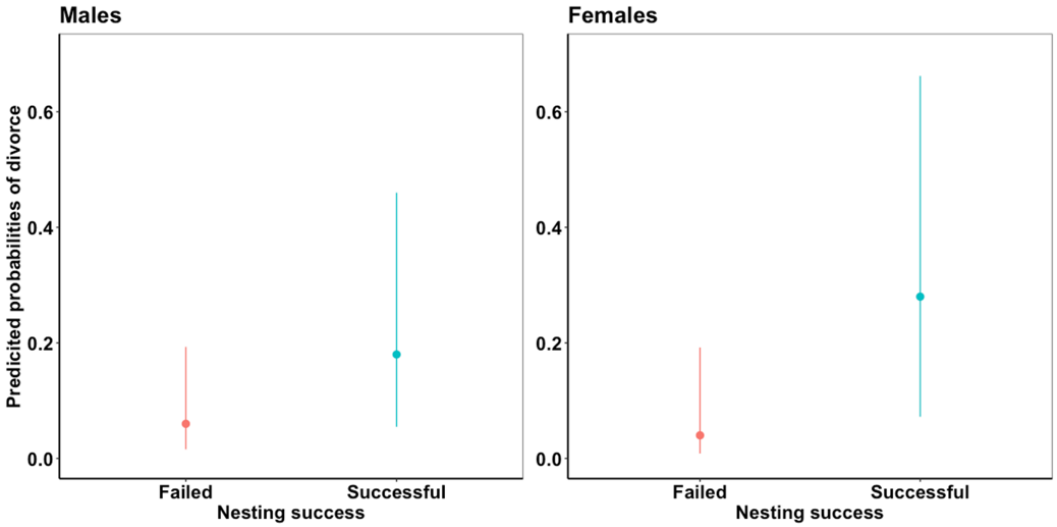

Figure 3.4 Mate fidelity in relation to nesting success in male and female plovers (see Table 3.3). Predicted probabilities of divorce, lower and upper 95% confidence intervals are shown.

Table 3.2 Mate fidelity in relation to ambient temperature, temperature variation, nesting success, body weight and sexual size dimorphism (SSD) in plover populations (generalised linear mixed models via Template Model Builder (TMB), including species and population as random effect variables). Separate models were constructed for males and females. SE = standard error. *P* values < 0.05 are emboldened.

Response variable	**Explanatory variables**	**Estimate**	**SE**	***z* value**	***P* value**
Mate fidelity rate in males (n = 70 years)					
	Intercept	8.26	2.50	3.32	**< 0.001**
	Ambient temperature	-0.11	0.52	-2.10	**0.04**
	Temperature variation	-2.46	1.37	-1.80	0.07
	Nesting success rate	-2.53	0.70	-3.59	**< 0.001**
	Body weight	-0.05	0.03	-1.52	0.13
	SSD	-64.34	36.63	-1.76	0.08
Mate fidelity rate in females (n = 73 years)					
	Intercept	5.87	4.01	1.46	0.14
	Ambient temperature	-0.05	0.08	-0.68	0.49
	Temperature variation	-1.27	1.87	-0.68	0.49
	Nesting success rate	-2.21	0.81	-2.72	**0.01**
	Body weight	-0.04	0.06	0.71	0.48
	SSD	-60.74	40.59	-1.50	0.13

Table 3.3 Mate fidelity in relation to nesting success and egg-laying date in plover populations. Generalized Linear mixed model with binomial family and including male/female ID, year, population and species as random effect variables. SE = standard error. *P* values < 0.05 are emboldened.

Response variable	Explanatory variables	Estimate	SE	*z* value	*P* value
Mate fidelity in males (n = 839 observations)					
	Nesting success (successful)	-1.56	0.67	-2.32	**0.02**
	Nesting success (failed)	-2.78	0.67	-4.14	**< 0.001**
	Egg-laying date	-0.18	0.21	-0.85	0.40
Mate fidelity in females (n = 921 observations)					
	Nesting success (successful)	-1.02	0.81	-1.26	0.21
	Nesting success (failed)	-3.14	0.83	-3.80	**< 0.001**
	Egg-laying date	-0.15	0.22	-0.68	0.49

3.3.4 Implications of mate fidelity

Divorced plovers (both males and females) produced significantly more hatchlings within years than those that retained their mate, although reproductive success (defined as the total number of hatchlings produced within a breeding year) was not different between divorced males and divorced females (Figure 3.5a; Table 3.4a). Divorced females dispersed greater distances than divorced males (breeding dispersal was defined as the straight-line distance in meters between an individual's successive nests within a breeding season); however, divorced males did not disperse farther than retained pairs (Figure 3.5b, Table 3.4b).

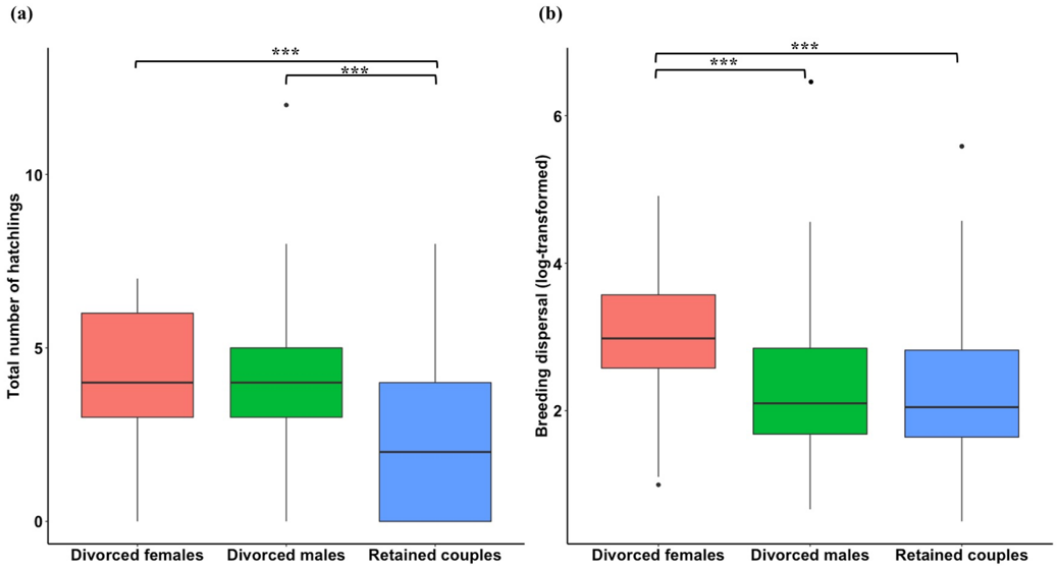

Figure 3.5 (a) Reproductive success and **(b)** breeding dispersal in relation to mate fidelity in plovers (see Tables 3.4a & b). Breeding dispersal was estimated in meters and log-transformed

(ln). Medians, upper and lower quartiles, as well as extreme values are shown, *** represents $P < 0.001$.

Table 3.4 (a) Comparison of reproductive success between divorced males, divorced females and retained pairs in plover populations (GLMM with Poisson error, maintaining individual ID, species and population as random effect variables, $\chi^2 = 39.76$, $df = 2$, $P < 0.001$, followed by *post-hoc* pairwise Tukey test). SE = standard error. *P* values < 0.05 are emboldened.

Comparison	Estimate	SE	z ratio	*P* value
Divorced females vs. divorced males	0. 06	0.09	0.62	0.81
Divorced females vs. retained pairs	0.41	0.07	5.92	**< 0.001**
Divorced males vs. retained pairs	0.35	0.09	4.06	**< 0.001**

(b) Comparison of breeding dispersal between divorced males, divorced females and retained pairs in plover populations (LMM via REML was fitted and maintained individual ID, species and population as random effect variables, $\chi^2 = 153.76$, $df = 2$, $P < 0.001$, followed by *post-hoc* pairwise Tukey test). SE = standard error. *P* values < 0.05 are emboldened.

Comparison	Estimate	SE	df	*t* ratio	*P* value
Divorced females vs. divorced males	0. 75	0.11	670	7.1	**< 0.001**
Divorced females vs. retained pairs	0.96	0.08	717	12.29	**< 0.001**
Divorced males vs. retained pairs	0.216	0.09	717	2.28	0.06

3.4 Discussion

Three major insights have emerged from our global study. First, our results indicate that mate fidelity rates within both sexes differ among populations, consistent with previous studies of plovers (Székely *et al*. 2006, Eberhart-Phillips 2019, Székely 2019). For instance, Kentish plover *Charadrius alexandrinus* populations in Europe and China commonly display serial polygamy with mostly females divorcing their mate soon after the chicks hatched (Lessells 1984, Székely and Lessells 1993, Amat *et al*. 1999); whereas the island population of Kentish plovers in Cape Verde is exclusively monogamous (Carmona-Isunza *et al*. 2015). The social mating system of all other plover species included in our study is monogamy except for the snowy plover which exhibit serial polygamy (Warriner *et al*. 1986, Eberhart-Phillips *et al*. 2018). Variation in mate fidelity between closely related species and populations is also common in primates, ungulates and fishes (Avise *et al*. 2002, Kappeler and van Schaik 2002, Bowyer *et al*. 2020).

Our study revealed that mate fidelity variation among plover populations is predicted by the ambient temperature, since populations in colder climates had higher mate fidelity rates than populations in warmer climates. We suggest that ambient temperature may largely influence the

mate fidelity rate by its association with an increase in the time available for breeding, and by increasing the chance that at least one breeding attempt will be successful (Blomqvist *et al*. 2001, Botero and Rubenstein 2012, Eberhart-Phillips 2019). For example, cold environments with short breeding seasons may limit the opportunity of multiple breeding with a new mate given that mate-search and courtship are time consuming, therefore, the best strategy is to re-mate immediately with same mate if there is a breeding failure (Green *et al*. 1977, Adkins-Regan and Tomaszycki 2007, Perfito *et al*. 2007, Johnson and Walters 2008). In contrast, mild environments with a prolonged breeding season enable a single parent to rear the offspring, and thus provide an opportunity for multiple breeding attempts for the other parent (Székely *et al*. 1999, Yasue and Dearden 2008, Halimubieke *et al*. 2019). However, it is also possible that temperature may influence mate fidelity rate by directly influencing other related behaviours or physiological processes (Botero and Rubenstein 2012). Ambient temperature appears to exert a weaker influence on mate fidelity of females than on males across populations, as males of populations from colder environments exhibit significantly higher mate fidelity rates than those from warmer environments. Further research is needed to clarify whether the different responses of males and females to environmental conditions are directly influenced by abiotic factors (Silva *et al*. 2007, Conrad *et al*. 2017, Brandt *et al*. 2018), or indirectly influenced by social environment (e.g. ASR; Eberhart-Phillips *et al*. 2018).

Our results also showed that mate fidelity rate exhibits no relationship with temperature variation between years, although studies suggest that annual fluctuations in temperature affect mating decisions in insect, reptile and mammalian species (Twiss *et al*. 2007, Olsson *et al*. 2011, Suzaki *et al*. 2018). We argue that temperature variation is probably a crude proxy of ambient environment fluctuation, since fluctuations in other abiotic environmental factors, for example precipitation and habitat quality, may also influence mate fidelity (Gilburn and Day 1994, Welch 2003, Candolin *et al*. 2007, Botero and Rubenstein 2012).

While we are unable to measure variation in the social environment across populations in this study, we also propose that aspects of the social environment, such as ASR, may also be strong contributor to mate fidelity variation. Recent studies show that ASR may deviate from 1:1 in a variety of organisms (Kosztolányi *et al*. 2011, Liker *et al*. 2013, Parra *et al*. 2014, Eberhart-Phillips *et al*. 2017). Variation in the ASR can alter the mating opportunities of breeding males and females, thus, influence divorcing and re-mating strategies (Liker *et al*. 2013, 2014, Eberhart-Phillips *et al*. 2018). The role of ASR influencing mating system variation in plovers and beyond will need to be revisited in the near future, although provisional studies of 4 populations suggest ASR does relate to parental care (Székely *et al*. 1999, Eberhart-Phillips *et al*. 2017, Eberhart-Phillips *et al*. 2018).

The second major insight of our study is that breeding success is an important predictor of divorce. At the population level, populations with high nesting success rates have lower mate fidelity rates compared to populations with low nesting success rates. Consistently, individuals were more likely to divorce after clutches hatched rather than when they did not hatch, and failed breeders

typically re-nested with the same partner in each population with the possible exception of white-fronted plovers, *Charadrius marginatus* in which divorce was not observed. As a consequence, divorced individuals, counterintuitively, rear more offspring compared to faithful individuals (Figure 3.5a). This finding does not support the "*incompatibility hypothesis*" (Kempenaers *et al*. 1998, Pyle *et al*. 2001), which predicts that breeding pairs with low breeding success should be more likely to divorce (Handel and Gill 2000, Adkins-Regan and Tomaszycki 2007). We posit that by divorcing and rapidly changing partners, while simultaneously deserting their current brood, individuals can produce more offspring within a limited season to maximize their reproductive success. Why would divorce be beneficial? (i) Offspring mortality is generally high and stochastic in shorebirds; thus, individuals may need to reproduce several times within a breeding season to produce at least some offspring (Cruz-López *et al*. 2017). (ii) Chicks are precocial and only require modest care (Székely *et al*. 1999), which provides the opportunity for one parent to terminate care and initiate a new clutch with another mate (Székely *et al*. 1996, McNamara *et al*. 1999, Houston *et al*. 2013). In contrast, mate retention was more likely after breeding failure. We suggest re-mating with the previous partner is the fastest way for both pair members to breed again ("fast-track hypothesis"; Zann 1994, Fowler 1995, Adkins-Regan and Tomaszycki 2007, Perfito *et al*. 2007); Breeding failure is related to partner compatibility in insects and mammalian species (de Crespigny *et al*. 2008, Schwensow *et al*. 2008), although we suspect that nest failure in shorebirds is majorly driven by predation (Fraga and Amat 1996, Ferreira-Rodriguez and Pombal 2018, Kubelka *et al*. 2018), thus we presume the role of partner compatibility in divorce is weaker compared to extrinsic forces like predation. Therefore, re-mating seems more important than changing partners and risking not finding a new mate.

Third, we found that mate fidelity is related to breeding dispersal. After divorce, female plovers disperse significantly farther than males. Sex-biased dispersal has been well-documented in invertebrates, reptiles, birds, and mammals, and it is proposed to be related to mating system (Greenwood 1980, Clobert *et al*. 2001, Trochet *et al*. 2016). For example, many mammals are socially polygynous, males do not participate in parental care, females rely on home ranges with resources to successfully rear offspring, therefore, male-biased dispersal is expected. Whereas in birds, which are typically socially monogamous, males demonstrate territorial defence behaviour because high quality breeding sites provide good resources and opportunities for successful breeding, thus reducing male's tendency to move large distance between breeding attempts (Greenwood and Harvey 1982, Sandercock *et al*. 2000, Liu and Zhang 2008, Vegvari *et al*. 2018). Our result follows the general pattern of female-biased breeding dispersal observed in most bird species including shorebirds (Lloyd 2008, Pearson and Colwell 2014). Additionally, we propose that for polygamous populations there is an additional reason: females have the opportunity to desert the brood and seek a new mate from within a wide geographical area (D'Urban Jackson *et al*. 2017).

Taken together, our results illustrate that (i) mating decisions are associated with the abiotic environmental conditions; (ii) birds that divorce and desert their broods generally attain higher breeding success than individuals that retained their mates; and (iii) the asymmetric mating opportunities of males and females result in different spatial dispersal patterns. Our results support the proposition that divorce is a strategy employed to improve reproductive success (Lessells 1984, McNamara and Forslund 1996, Halimubieke *et al*. 2019). We suggest that divorce is an adaptive response to environmental constraints (e.g. limited breeding time), life history traits (e.g. low survival rate of the young, uniparental care) and population demography (e.g. biased ASR). We call for further studies to build upon our research framework by augmenting these analyses with other more environmental variables (e.g. precipitation) and incorporating information on the social environment (e.g. ASR) and broader scope of life history traits (e.g. survival rate, longevity). In addition, we encourage the development of theoretical models investigating the influence of ecological/ social environment and life history on the evolution of breeding systems.

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Ethical statement

This study did not involve any manipulation experiments, and all methods were carried out in accordance with relevant guidelines and regulations of each country in which it was performed. Fieldwork and bird-ringing procedures were authorized by relevant authorities: Hungary (Environmental Ministry and Kiskunság National Park); Australia (Deakin University Animal Welfare Committee Permits B02-2012, B20-2014 and B10-2016, State Government Permits 10006205, 10007918 and 10007241 and Australian Bird and Bat Banding Scheme (ABBBS) Authorities 1763, 3271 and 3033.); Mexico (#SGPA/ DGVS/01717/10, #SGPA/DGVS/01367/11), Spain (Ministry of Environment #660117); California (U.S. Fish and Wildlife (USFWS)

#TE807078 and U. S. Geological Survey (USGS) #09316); Great Plain, USA (the U.S. Geological Survey Bird Banding Laboratory with Federal Master Bander permit #21446 with threatened and endangered species endorsements, Federal Threatened and Endangered Species handling permit#TE103272-3, and IACUC protocol #14-003.); China (Hebei Forestry Bureau); South Africa (Cape Nature and SAFRING); Cape Verde (Directorate Geral Ambiente); Turkey (Turkish Ministry of National Parks, Tuzla Municipality and Governor of Karatas, Mr. E. Karakaya).

Conflict of interest

The authors declare that they have no competing interests.

Date availability

Relevant data is available from GitHub (https://github.com/narhulan29/charadriusplovers)

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Supplementary Materials

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Appendix S1. Description of the Bayesian modelling framework.

To test how phylogenetic relatedness might influence our results we constructed Bayesian MCMCglmm models implemented in R package 'MCMCglmm' with categorical trait distribution[1].The phylogenetic tree was obtained from dos Remedios *et al*.[2]. Since prior information about parameter distribution was not available, we used non-informative priors. All models were run for 500,000 times with a burn-in of 1,000 and a thinning interval of 500. Phylogenetic effect was tested by removing the phylogeny regarding the species random term, while keeping the latter random term in the model. Species effect was tested by removing phylogeny information and the species random term. Phylogenetic signal was calculated for all four initial models.

Prior specifications are given below, where *n* stands for the number of fixed parameters estimated in each of the models, *Species* is a phylogenetic tree representing the evolutionary history of the 8 *Charadrius* species included in this study. Number of *G* structure elements in the prior was modified according to the number of random factors in each model.

```
Prior<- list (G = list (G1 = list (V = 1, nu = 1000, alpha.mu = 0, alpha.V = 1),
                        G2 = list (V = 1, nu = 1000, alpha.mu = 0, alpha.V = 1),
                        ... Gn = list (V = 1, nu = 1000, alpha.mu = 0, alpha.V = 1),
```

```
        R = list (V = n, fix = TRUE))
inv.phylo <-inverseA (Species,nodes = "TIPS",scale = TRUE)
phylogenetic signal <- model$VCV[, "species"]/(model$VCV[, "species"] + model$VCV[,
"units"])

model 1<-MCMCglmm (mate fidelity rate ~ ambient temperature + temperature variation +
nesting success rate + average body weight + SSD,
        random = ~ species + population,
        family = "gaussian",
        ginverse = list (Species = inv.phylo$Ainv),
        nitt = 500000, burnin = 1,000, thin = 500,
        prior = Prior, singular.ok = T)
phylogenetic signal: 0.04

model 2 <-MCMCglmm (mate fidelity rate ~ ambient temperature + temperature variation +
nesting success rate + average body weight + SSD,
        random = ~ species + population,
        family = "gaussian",
        ginverse = list (Species = inv.phylo$Ainv),
        nitt = 500000, burnin = 1,000, thin =500,
        prior = Prior, singular.ok = T)
phylogenetic signal: 0.04

model 3 <-MCMCglmm (mate fidelity ~ nesting success + egg-laying date,
        random = ~ year + male_ID + species + population,
        family = "categorical",
        ginverse = list (Species = inv.phylo$Ainv),
        nitt = 1,000,000, burnin = 1,000, thin = 500,
        prior = Prior, singular.ok = T)
phylogenetic signal: 0.38

model4 <-MCMCglmm (mate fidelity ~ nesting_success + egg_laying_date,
        random = ~ year + female_ID + species + population,
        family = "categorical",
        ginverse = list (Species = inv.phylo$Ainv),
        nitt = 1,000,000, burnin = 1,000, thin = 500,
        prior = Prior, singular.ok = T)
```

phylogenetic signal: 0.36

```
model 5<-MCMCglmm(mate fidelity ~ daily temperature
        random = ~ year + male_ID + species + population,
        family = "categorical",
        ginverse = list (Species = inv.phylo$Ainv),
        nitt= 1,000,000, burnin = 1,000, thin= 500,
        prior = Prior, singular.ok = T)
```

phylogenetic signal: 0.67

```
model 6<-MCMCglmm(mate fidelity ~ daily temperature
        random = ~ year + female_ID + species + population,
        family = "categorical",
        ginverse = list (Species = inv.phylo$Ainv),
        nitt = 1,000,000, burnin = 1,000, thin = 500,
        prior = Prior, singular.ok = T)
```

phylogenetic signal: 0.61

```
model 7<-MCMCglmm(mate fidelity ~ body weight
        random = ~ year + male_ID + species + population,
        family = "categorical",
        ginverse = list (Species = inv.phylo$Ainv),
        nitt = 1,000,000, burnin = 1,000, thin = 500,
        prior = Prior, singular.ok = T)
```

phylogenetic signal: 0.28

```
model 8<-MCMCglmm(mate fidelity ~ body weight
        random = ~ year + female_ID + species + population,
        family = "categorical",
        ginverse = list (Species = inv.phylo$Ainv),
        nitt = 1,000,000, burnin = 1,000, thin = 500,
        prior = Prior, singular.ok = T)
```

phylogenetic signal: 0.35

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Appendix S2. Supplementary references for Table 3.1

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Table S1. Mate fidelity in relation to daily temperature and body weight within breeding years in plover populations. Generalised linear mixed model with binomial family and including male/female ID, year, population and species as random effect variables. SE = standard error. *P* values < 0.05 are emboldened.

Response variable	Explanatory variable	Estimate	SE	*z* value	*p* value
Mate fidelity in males (n = 788 observations)					
	Intercept	-2.58	1.12	-2.17	**0.03**
	Daily temperature	-0.03	0.03	-0.82	0.41
Mate fidelity in females (n = 776 observations)					
	Intercept	-3.86	1.78	-2.16	**0.03**
	Daily temperature	0.01	0.03	0.40	0.69
Mate fidelity in males (n = 136 observations)					
	Intercept	1.12	1.67	0.67	0.50
	Body weight	-0.03	0.04	-0.95	0.34
Mate fidelity in females (n = 193 observations)					
	Intercept	2.51	1.79	1.40	0.16
	Body weight	-0.06	0.04	-1.55	0.12

Table S2. Summary description of the distances of weather stations from study sites (km) used in the study.

English name	Population	Distance of weather stations from study site (km)
Kentish plover	Bohai Bay (China)	29
Kentish plover	Great Hungarian Plain (Hungary)	19.2
Kentish plover	Senigallia (Italy)	202
Kentish plover	Maio Island (Cape Verde)	181
Kentish plover	Tuzla Lake (Turkey)	204
Kentish plover	Llobregat Delta (Spain)	4.5
piping plover	New Jersey (USA)	21
piping plover	Great Plain (USA)	48.1
red-capped plover	Altona (Cheetham) Saltworks (Australia)	5.1
snowy plover	Ceuta Bay (Mexico)	12
two-banded plover	Sea Lion Island (Falklands)	5
killdeer	Honey Lake, California (USA)	21.3
white-fronted plover	Cape Peninsula (South Africa)	50
Malaysian plover	Prachuap Khiri Khan (Thailand)	40.2

Chapter 4 | How do biases in sex ratio and disease characteristics affect the spread of sexually transmitted infections?

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This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.

Author's Contributions

BA, NH and AP designed the model;

BA and NH analysed and interpreted the result;

NH and AP co-wrote the first draft;

All authors contributed to revisions of the manuscript.

Abstract

The epidemiology of sexually transmitted infections (STIs) is inherently linked to host mating dynamics. Studies across many taxa show that adult sex ratio, a major determinant of host mating dynamics, is often skewed - sometimes strongly - toward males or females. However, few predictions exist for the effects of skewed sex ratio on STI epidemiology, and none when coupled with sex biased disease characteristics. Here we use mathematical modelling to examine how interactions between sex ratio and disease characteristics affect STI prevalence in males and females. Notably, we find that while overall disease prevalence peaks at equal sex ratios, prevalence per sex peaks at skewed sex ratios. Furthermore, disease characteristics, sex-biased or not, drive predictable differences in male and female STI prevalence as sex ratio varies, with higher transmission and lower virulence generally increasing differences between the sexes for a given sex ratio. These findings may be due to a balance between increased per-capita mating in the less common sex, and a reduction in mating rate - hence disease prevalence - at the population level. Our work reveals new insights into how STI prevalence in males and females depends on a complex interaction between host population sex ratio and disease characteristics.

Keywords

sex ratio, sexual transmission, disease prevalence, STI, mating dynamics

4.1 Introduction

Sexually transmitted infections (STIs) – defined as any pathogen that is transmitted during copulation – are ubiquitous in the animal kingdom, often causing chronic infections with low recovery rates and reduced reproductive success, even sterility (Lockhart *et al*. 1996, Ashby *et al*. 2019). STIs typically exhibit contrasting epidemiological dynamics to non-STIs, with transmission likely to be frequency- rather than density-dependent (i.e. the number of sexual contacts per capita is invariant to population size). Another major difference is that STIs are primarily transmitted between the sexes (especially in non-human populations), which creates a natural bipartite network where the population is split into two disjoint sets (males and females) and connections only exist between individuals from distinct sets. In contrast, non-STI transmission networks are generally not bipartite. Sex ratio, which can vary greatly in non-human populations (Dyson and Hurst 2004, Liker *et al*. 2013, Székely *et al*. 2014, Bonnet *et al*. 2016), will therefore likely have a profound impact on STI epidemiology compared to non-STI epidemiology, as it disrupts the structure of the contact network (Hurst *et al*. 1997, Ryder *et al*. 2014). Growing numbers of theoretical studies and empirical studies in humans are shedding light on the relationship between host mating system structure and STI dynamics, showing that polygamous sex exhibits much lower levels of infection than the monogamous sex (Anderson and May 1991, Thrall et al. 1997, Thrall et al. 2000, Eames and Keeling 2002, Ashby and Gupta 2013,) However, the STIs transmission dynamics in wild populations, especially birds, as well as the implications of variation in sex ratio for STI epidemiology are not well understood.

Evidence has shown that sex ratio has a crucial role in mating system variation in wild populations (Székely *et al*. 2014, Eberhart-Phillips *et al*. 2017). Sex ratio can vary at different stages of the life cycle, with the ratios of males to females at conception, at birth and during adult life (termed primary, secondary, and tertiary or adult sex ratio, respectively). Recent studies show that adult sex ratio may deviate from 1:1 in a variety of organisms: butterflies, reptiles and birds often have male-biased sex ratios with up to 90% of the population being male, whereas female-biased adult sex ratios are common in insects and mammals, with up to 100 females for every male (Dyson and Hurst 2004, Liker *et al*. 2013, Székely *et al*. 2014, Bonnet *et al*. 2016). Variation in the adult sex ratio can alter the mating opportunities of breeding males and females and may select for different mating systems (Emlen and Oring 1977, Rankin and Kokko 2007), the latter has significant consequences for STI dynamics (Anderson *et al*. 1989, Anderson 1991, Thrall and Antonovics 1997, Thrall *et al*. 1998). Interestingly, *Wolbachia* bacteria (transmitted vertically rather than sexually in insect hosts) are known to distort the birth sex ratio, as it is maternally inherited and kills male embryos, consequently, disrupt the mating dynamics of their hosts (Hurst *et al*. 1997, Jiggins *et al*. 2000, Charlat *et al*. 2007).

Adult sex ratio is clearly crucial for mating dynamics and hence STI transmission, but it is shaped by multiple factors, including primary and secondary sex ratio as well as differential survival

during juvenile and adult stages (Ancona *et al*. 2017). STIs may cause differential mortality between the sexes, potentially increasing or decreasing the ratio of adult males to females, causing feedback between STI transmission and sex ratio (Székely *et al*. 2014). In general, theory predicts that the rarer sex in the population should exhibit higher STI prevalence since they have a higher per capita mating rate, all else being equal (Ryder *et al*. 2014). For example, two-spot ladybirds *Adalia bipunctata* exhibit male-biased patterns of STI prevalence in female-skewed populations (Ryder *et al*. 2014). A follow-up experimental study by Pastok *et al*. (Pastok 2015) established that the presence of male-killing bacteria at high prevalence skews the sex ratio towards a female-biased population, and results in male-biased STI prevalence. Together, these studies suggest that there are likely to be complex interactions between STIs and adult sex ratios. However, the extent to which STI prevalence in males and females depends on sex ratio and disease characteristics, which may vary between the sexes, has yet to be explored.

Many pathogens – both STIs and non-STIs - show sex bias in disease characteristics (e.g. transmissibility, virulence), which can influence disease dynamics (Sheridan *et al*. 2000, Miller *et al*. 2007). Two main hypotheses have been proposed to explain sex-biased disease characteristics. The *physiological hypothesis* emphasises that the interactions between sex hormones and the immune system render one sex more susceptible to infection and disease (Zuk and McKean 1996). For example, in mammals, males have consistently weaker immune competence than females, and this correlates with male-biased disease prevalence, mortality, and female-biased adult sex ratio (Klein and Roberts 2010, Metcalf and Graham 2018, Lemaître *et al*. 2020) On the other hand, the *behavioural hypothesis* posits that sexual differences in behaviour may cause sex-specific exposure to pathogens (Brei and Fish 2003). For instance, infections by arthropods, helminths and unicellular parasites are often male-biased in mammals but not in birds (Moore and Wilson 2002). Sex differences are expected, since males tend to be more active in reproductive behaviours such as combat for females, territorial defence and foraging, due to more intense sexual selection in males than females, therefore increasing the chance of exposure to pathogens. In human populations, gender-related behavioural differences may render one sex more exposed to certain contagions (STIs or non-STIs). For example, Ebola haemorrhagic fever outbreak that occurred in the Congo and Gabon in 2001-2002 suggested more men than women were infected during the early stages of the outbreak because men spent more time working away from home, where they were more likely to come into contact with wild animals (World Health Organization 2007). Regardless of whether sex biases in disease characteristics are due to physiological or behavioural differences between males and females, there are likely to be important implications for variation in disease prevalence between the sexes.

In the context of STIs, current empirical research in sex-biased disease characteristics is mainly focused on humans. Some studies have established that there is a higher risk of transmission of certain STIs from men to women than vice versa due to STIs being present in ejaculate (Coombs

et al. 2003, Panchanadeswaran *et al*. 2006); another study shows that human T-lymphotropic virus exhibits male-biased virulence, potentially because transmissions occur during pregnancy, birth or breast-feeding, and so selection has favoured lower virulence in women (Úbeda and Jansen 2016). Empirical studies of sex-biased STI characteristics in animals are limited, but mathematical models have shown how sex-biased disease characteristics influence transmission dynamics (reviewed in (Ashby and Gupta 2013), also see (Anderson and May 1991)). However, the interplay between sex ratio and disease-characteristics (especially when these are sex-biased) and their effects on STI prevalence have yet to be investigated. Here we use mathematical modelling to examine the effects of biases in population sex ratio and disease characteristics on STI epidemiology. Specifically, we investigate how (1) variation in population sex ratio; (2) sex-biases in transmission and disease-associated mortality (virulence) in adults; and (3) the interactions between sex ratio and disease characteristics, impact on STI prevalence in males and females.

4.2 Methods

We model the dynamics of an STI in a randomly mixing population. The epidemiological and mating dynamics are described by the following system of ordinary differential equations:

$$\frac{dS_i}{dt} = \underbrace{b_i(S_M, S_F, I_M, I_F)}_{Births} - \underbrace{\frac{\beta_i c S_i I_j}{N}}_{Infections} - \underbrace{dS_i}_{Natural\ mortality} + \underbrace{\gamma I_i}_{Recovery} \quad (1)$$

$$\frac{dI_i}{dt} = \underbrace{\frac{\beta_i c S_i I_j}{N}}_{Infections} - \underbrace{dI_i}_{Natural\ mortality} - \underbrace{\alpha_i I_i}_{Mortality\ virulence} - \underbrace{\gamma I_i}_{Recovery} \quad (2)$$

where $i, j \in \{M,F\}$, $i \neq j$, correspond to males and females. The birth rates for each sex are

$$b_M(S_M, S_F, I_M, I_F) = \frac{bcr(1-qN)}{N}(S_M + I_M)(S_F + I_F), \quad (3)$$

$$b_F(S_M, S_F, I_M, I_F) = \frac{bc(1-r)(1-qN)}{N}(S_M + I_M)(S_F + I_F), \quad (4)$$

and: S_i and I_i (equivalently, S_j or I_j), are the number of susceptible and infected individuals, respectively, in each sex; $N = S_M + S_F + I_M + I_F$ is the total population size; r is the sex ratio at birth (the proportion of offspring that are male), with $0 \leq r \leq 1$; $c \geq 0$ is the pairwise rate at which males and females mate given frequency-dependent contact; q represents the strength of density-dependence on births; d is the natural mortality rate; α_i is the disease-associated mortality rate (virulence) in sex i; β_i is the transmission rate from sex j to sex i per sexual contact; γ is the recovery rate. The probability that a susceptible female mates with an infected male (similarly, for the reverse

scenario) is equal to $\frac{I_M}{S_M+I_M}$, which is obtained by dividing the mating rate with infected males $\frac{cS_FI_M}{N}$ by the total mating rate for susceptible females $\frac{cS_F(S_M+I_M)}{N}$.

For simplicity, we assume that: (i) both sexes have the same recovery rate from the STI, but will not develop immunity, which means they are still susceptible to STI after recovery; (ii) both sexes have the same natural mortality rate; (iii) the per-capita mating rate is frequency-dependent, which means that larger populations do not have a higher per-capita mating rate than smaller populations; (iv) there is no structuring or choice in the mating system (mating is random); (v) there is no juvenile period; and (vi) there is no effect of parental care on the population dynamics.

The disease-free equilibrium $(S_M, S_F, I_M, I_F) = (S_M^*, S_F^*, 0, 0)$, of this system occurs at:

$$S_M^* = \frac{bcr(1-r)-d}{bcq(1-r)} \tag{5}$$

$$S_F^* = \frac{bcr(1-r)-d}{bcqr} \tag{6}$$

A newly introduced STI will spread in a susceptible population when the basic reproductive ratio, R_0 is greater than 1 (R_0 is calculated as the maximum eigenvalue of the Jacobian of the system, evaluated at the disease-free equilibrium), where:

$$R_0 = \frac{c}{S_F^*+S_M^*}\sqrt{\frac{\beta_M\beta_F S_M^* S_F^*}{(d+\gamma+\alpha_M)(d+\gamma+\alpha_F)}} \tag{7}$$

In order to guarantee that $R_0 > 1$, we require that $\frac{1}{2} - \psi < r < \frac{1}{2} + \psi$, where $\psi = \sqrt{\frac{1}{4} - \frac{(d+\gamma+\alpha_M+\alpha_F)(d+\gamma)}{\beta_M\beta_F c^2}}$. To test the interactions between sex ratio and STI prevalence in males and females, we start with no sex bias in STI characteristics. This means that males and females are equally susceptible to the pathogen and the mortality caused by the pathogen is same in both sexes ($\alpha_M=\alpha_F=\alpha$; $\beta_M=\beta_F=\beta$). STI prevalence in each sex is given as the number of infected individuals in that sex divided by the total number of individuals in that sex (at equilibrium, as indicated by asterisks):

$$D_M = \frac{I_M^*}{S_M^* + I_M^*} \tag{8}$$

$$D_F = \frac{I_F^*}{S_F^* + I_F^*} \tag{9}$$

To examine the impact of sex-biased disease characteristics on the prevalence of STIs in males and females, we fix female susceptibility (β_F) and disease-related mortality (α_F) and vary the corresponding parameters in males. The model is symmetric up to labelling, so the results are analogous for the converse scenario. We primarily use numerical analysis to determine disease prevalence as the endemic equilibrium of equations 1-2 is mostly intractable to algebraic analysis.

4.3 Results

4.3.1 Non-sex-biased disease characteristics

We begin by exploring how sex ratio impacts on STI prevalence in males and females when there are no sex-biases in disease characteristics (i.e. $\alpha_M = \alpha_F = \alpha$; $\beta_M = \beta_F = \beta$). Consistent with previous theoretical and empirical work (Hurst *et al*. 1997, Ryder *et al*. 2014), the less common sex exhibits higher STI prevalence at equilibrium (Figure 4.1). Intuitively, when there are no sex biases in disease characteristics, the overall prevalence of the STI is always maximised at an equal sex ratio ($r = 0.5$). However, both the extent to which STI prevalence differs between the sexes (i.e. the difference between the male and female curves in Figure 4.1a-b, shown in Figure 4.1c-d) and the point at which STI prevalence peaks in each sex (i.e. the sex ratio that maximises the male and female curves, indicated by circles in Figure 4.1a-b) depends on the transmission rate and virulence. Specifically, as the transmission rate increases or as the virulence decreases, the difference between male and female STI prevalence for a given sex ratio tends to increase, especially for more extreme sex ratios (Figure 4.1c-d). Furthermore, for higher transmission rates/lower virulence, the sex ratio at birth for which disease prevalence peaks becomes more extreme. Thus, while overall STI prevalence is qualitatively unchanged by variation in the transmission rate and virulence (i.e. it always peaks at $r = 0.5$), STI prevalence per sex is highly sensitive to disease characteristics.

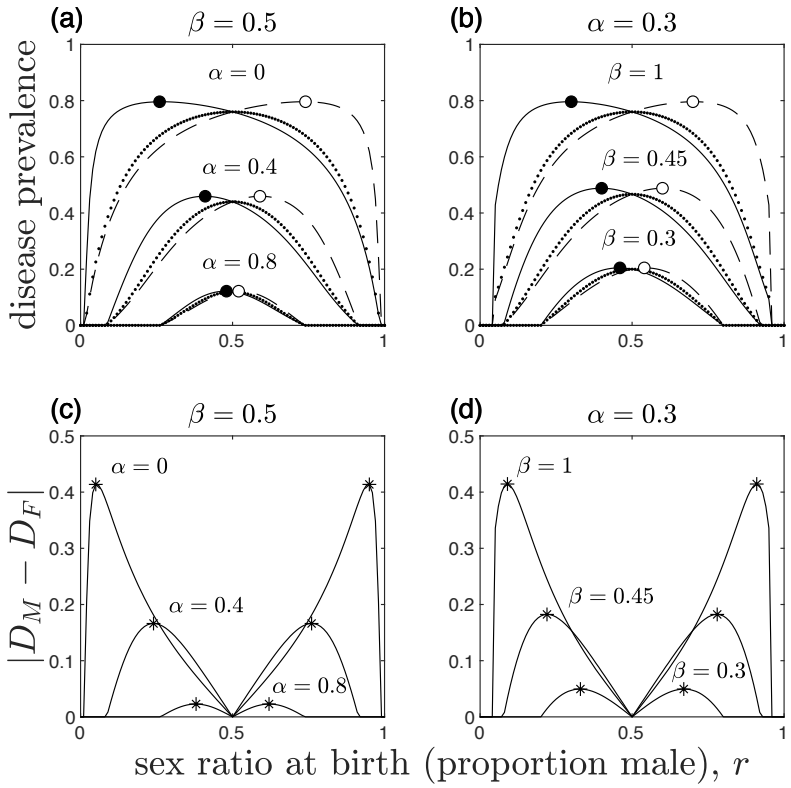

Figure 4.1 Effects of sex ratio at birth (proportion male), r, on STI prevalence when there are no sex biases in disease characteristics: (a, c) variation in disease-related mortality, $\alpha_M = \alpha_F = \alpha$; (b, d) variation in transmission probability, $\beta_M = \beta_F = \beta$. As disease-related mortality increases or the transmission probability decreases, overall STI prevalence decreases, and the difference between male and female STI prevalence for a given sex ratio decreases. (a, b) STI prevalence in males (solid line), females (dashed line), and overall (dotted line). Markers show where STI prevalence peaks in males (black) and females (white). (c, d) Absolute difference in STI prevalence between males (D_M) and females (D_F), corresponding to the absolute difference between the solid and dashed lines in (a) & (b), with markers showing where the absolute difference peaks. Other parameters are held constant throughout to ensure R_0 is greater than 1: $\delta = 0.1; \gamma = 0.2; c = 5; b = 1; q = 0.001$.

We can see this analytically for variation in the transmission rate if we make the following simplifying assumptions: (1) there is no disease-associated mortality ($\alpha_M = \alpha_F = 0$); (2) the disease transmission rates are equal ($\beta_M = \beta_F = \beta$); and (3) there is no recovery ($\gamma = 0$). These assumptions ensure that the adult sex ratio is equal to the sex ratio at birth, which greatly simplifies the analysis. First, we must calculate the equilibrium population size, N^*. Let $M = S_M + I_M$ be the number of males in the population and $F = S_F + I_F$, be the number of females in the population.

Then at equilibrium, $M = sN^*$, and $F = (1-r)N^*$. Now, $\frac{dN}{dt} = \frac{dS_M}{dt} + \frac{dS_F}{dt} + \frac{dI_M}{dt} + \frac{dI_F}{dt}$, and evaluating this gives

$$\frac{dN}{dt} = \frac{bc(1-qN)MF}{N} - dN \tag{10}$$

Substituting in $M = sN^*$ and $F = (1-r)N^*$, and solving $\frac{dN}{dt} = 0$, the resulting (non-trivial) equilibrium population size is:

$$N^* = \frac{bcr(1-r) - d}{bcqr(1-r)} \tag{11}$$

We can now substitute the equilibrium population size into the full system of equations, giving the endemic equilibrium:

$$S_F^* = \frac{d(\beta c(1-r) + d)\zeta}{\beta bc^2 qr(1-r)} \tag{12}$$

$$S_M^* = \frac{S_F^*}{\zeta^2} \tag{13}$$

$$I_F^* = \frac{(\beta^2 c^2 r(1-r) - d^2)\zeta}{\beta bc^2 qr(1-r)} \tag{14}$$

$$I_M^* = \zeta I_F^* \tag{15}$$

where $\zeta = \frac{\beta c(1-r)-d}{\beta cs+d}$. The disease prevalence in males and females is therefore

$$D_M = \frac{I_M^*}{S_M^* + I_M^*} = \frac{\beta^2 c^2 r(1-r) - d^2}{\beta cr(\beta c(1-r) + d)} \tag{16}$$

$$D_F = \frac{I_F^*}{S_F^* + I_F^*} = \frac{\beta^2 c^2 r(1-r) - d^2}{\beta c(1-r)(\beta cr + d)} \tag{17}$$

To find the peak in disease prevalence with respect to r, one must differentiate equations 16-17 with respect to r and then find where they are equal to zero, i.e., find r_M^*, r_F^* such that $\frac{d}{dr}(D_F)|_{r=r^*} = 0$ and $\frac{d}{dr}(D_M)|_{r=r^*} = 0$. For females, STI prevalence peaks at

$$r_F^* = \frac{\beta c + d - \sqrt{\beta cd + 2d^2}}{\beta c} = 1 + \frac{d - \sqrt{\beta cd + 2d^2}}{\beta c} \tag{18}$$

and for males STI prevalence peaks at

$$r_M^* = \frac{-d + \sqrt{\beta cd + 2d^2}}{\beta c} \tag{19}$$

(Note that the other root lies outside $r \in [0,1]$). Taking derivatives with respect to β, we find that $\frac{dr_F^*}{d\beta} > 0$ and $\frac{dr_M^*}{d\beta} < 0$. Thus, as the transmission rate, β, increases, the sex ratio at which STI prevalence peaks in each sex always becomes more extreme.

4.3.2 Sex-biased disease characteristics

We now consider the effects of sex-biased disease characteristics on STI prevalence. Specifically, we investigate how STI prevalence (per sex and overall) varies when there are biases in (1) female-to-male (β_M) or male-to-female (β_F) transmission, and/or (2) disease-associated mortality (virulence) for males (α_M) or females (α_F). Since the only differences in the model between males and females are these parameters, without loss of generality we fix β_F and α_F whereas vary β_M and α_M accordingly.

Sex differences in transmission rate and in virulence influence disease prevalence in both sexes, although the extent of their influence on males and females depends on sex ratio. A higher female-to-male transmission rate relative to the male-to-female transmission rate raises disease prevalence in both sexes, although interestingly the influence of sex ratio differs between the sexes. With greater female-to-male transmission, the disease prevalence peak shifts toward male-biased sex ratios in females whereas for males there is relatively little change (e.g. Figure 4.2a-c; Figure 4.3a-c). Similarly, lower disease-associated mortality in males relative to females also universally increases disease prevalence and shifts the peaks of the disease prevalence curves. However, lower values of α_M generally cause the disease prevalence peaks in each sex to shift towards lower values of r, with greater effects on male than female STI prevalence (e.g. Figure 4.2b, e, h; Figure 4.3d-f).

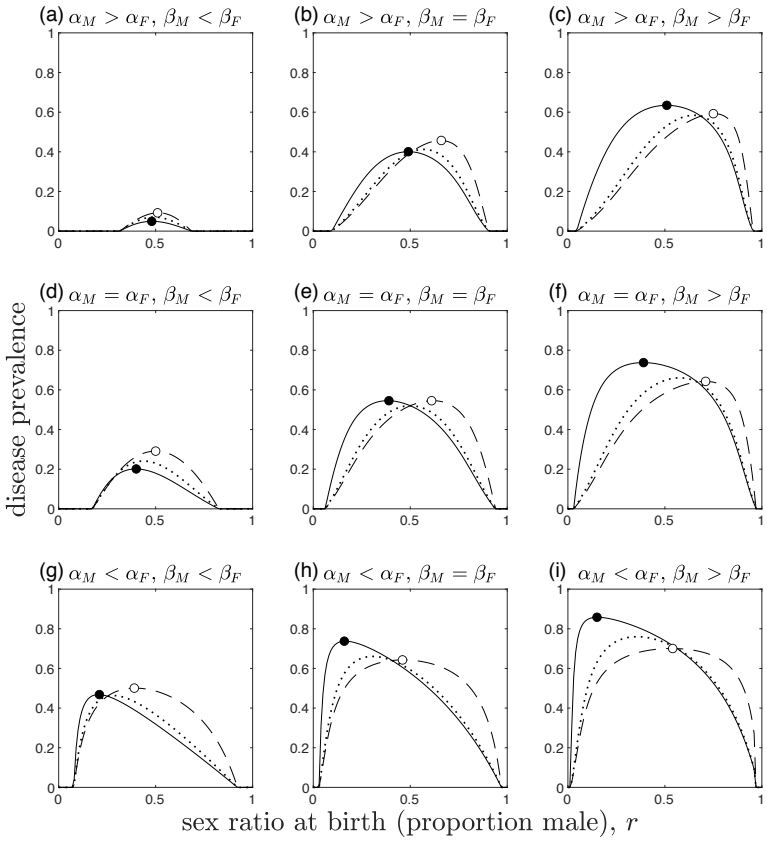

Figure 4.2 STI prevalence in males (solid line), females (dashed line), and overall (dotted line) as a function of the sex ratio at birth (proportion male), r, when there are sex biases in STI transmission rate and virulence. The parameters $\beta_F = 0.5$ and $\alpha_F = 0.3$ are held constant throughout, while $\beta_M \in \{0.2, 0.5, 1\}$ and $\alpha_M \in \{0, 0.3, 0.6\}$ vary between panels. Other parameters as described in Figure 4.1.

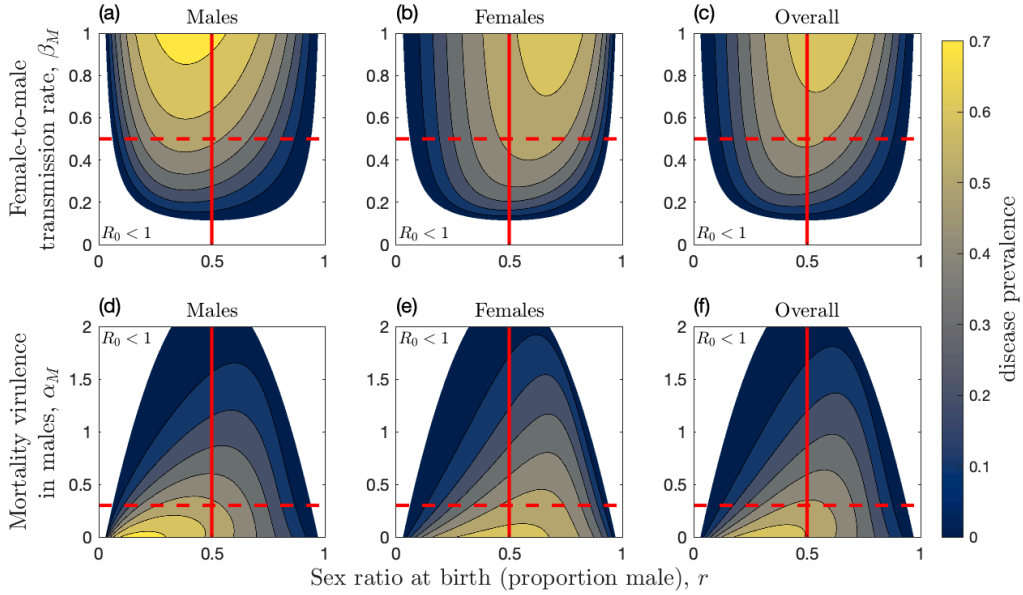

Figure 4.3 STI prevalence in (a, d) males, (b, e) females, and (c, f) overall as a function of the sex ratio at birth and: (a-c) female-to-male transmission rate (β_M); (d-f) mortality virulence in males (α_M). The red lines show when the sex ratio at birth is equal (solid) and when there is no sex bias in disease characteristics (dashed): (a-c) $\beta_M = \beta_F = 0.5$; (d-f) $\alpha_M = \alpha_F = 0.3$. The white region in each panel corresponds to conditions where the STI is unable to persist ($R_0 < 1$). The parameters $\beta_F = 0.5$ and $\alpha_F = 0.3$ are held constant throughout, Other parameters as described in Figure 4.1.

We see consistent patterns when transmission and virulence are varied simultaneously (Figure 4.4). By measuring the sex ratio at birth, r, for which disease prevalence peaks, we analyse how the interaction between transmission, virulence, and sex ratio leads to changes in disease prevalence. When disease-associated mortality in males is relatively low ($\alpha_M < \alpha_F$), variation in the female-to-male transmission rate (β_M) has little impact on the sex ratio where disease prevalence peaks in males (Figure 4.4a) and overall (Figure 4.4c), but the effect in females is much more pronounced, with the skew generally increasing for greater β_M (Figure 4.4b). When disease-associated mortality in males is relatively high ($\alpha_M > \alpha_F$), the sex ratio at birth where disease prevalence peaks in males and overall also increases with β_M, but to a lesser extent than in females. It is also clear from the contours in Figure 4.4 that variation in either transmission or virulence can cause non-monotonic changes in the disease prevalence curves. For example, the horizontal and vertical red lines in Figure 4.4 (indicating $\beta_M = \beta_F$ or $\alpha_M = \alpha_F$) intersect some of the contours twice, which means that varying either α_M or β_M can cause a non-monotonic change in the skew of the disease prevalence curves. Together, these results show that interactions between sex-biased disease characteristics and sex ratio can lead to major changes in STI prevalence within each sex and overall, and that the differences between male and female STI prevalence can be exacerbated by greater transmission rates or lower mortality rates, in one or both sexes.

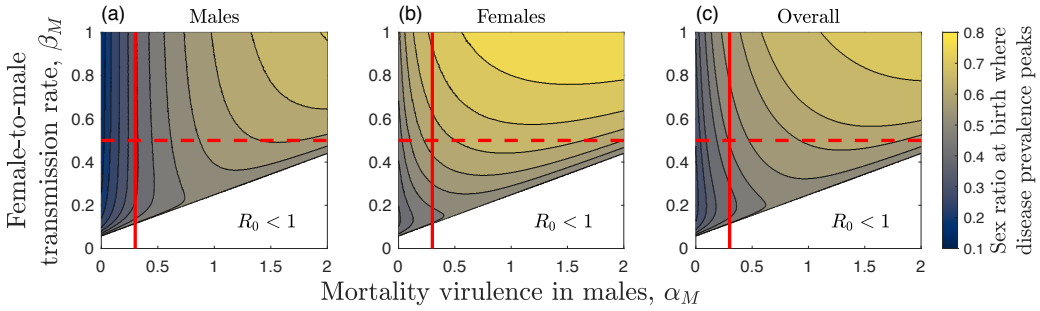

Figure 4.4 The sex ratio at birth for which disease prevalence peaks in: (a) males, (b) females, and (c) overall, as a function of disease-associated mortality for males (α_M) and female-to-male transmission rate (β_M). The red lines show when there is no sex bias in transmission (β_M=β_F=0.5; dashed) and when there is no sex bias in virulence (α_M=α_F=0.3; solid). The white region in the

bottom right corner of each panel corresponds to conditions where the STI is unable to persist ($R_0 < 1$). Other parameters as described in Figure 4.1.

4.4 Discussion

Studies of STI epidemiology emphasise the importance of the host mating system for STI dynamics (Thrall and Antonovics 1997, Thrall *et al*. 1997, Thrall *et al*. 2000, Ashby and Gupta 2013, Ryder *et al*. 2014, Pastok 2015). Since sex ratio influences mating systems, here we used mathematical modelling to examine the interaction between sex ratio and disease-characteristics, and their effects on STI prevalence in males and females. At a fundamental level, our results reveal how sex differences in STI prevalence within and between populations may be explained by both variation in sex ratio and disease characteristics. Moreover, our model shows that disease-characteristics, whether they be sex-biased or not, can increase or decrease differences in male and female STI prevalence.

In a population with a skewed adult sex ratio, the less common sex has more prospective mates and a higher per-capita mating rate than the more common sex. This pattern can be seen in human and non-human societies; for instance, in human communities with a male- skewed adult sex ratio, men are more likely to purchase sex and women are more likely to have multiple sex partners (Tucker *et al*. 2005, Bien *et al*. 2013). Consistently, in bird populations mating opportunities are related to adult sex ratios: in male-biased adult sex ratio, females have higher mating opportunities than males, whereas in female-skewed populations males re-mate faster than the females (Székely *et al*. 1999, Parra *et al*. 2014, Carmona-Isunza *et al*. 2015). Hence, with high variance in mating success, many individuals of the more common sex may remain unmated or have a low mating rate, whereas the less common sex has a higher mating rate and hence greater exposure to infectious partners. Thus, equilibrium disease prevalence in the more common sex is expected to be lower than in the rarer sex, consistent with previous theoretical and empirical studies (Hurst *et al*. 1997, Thrall *et al*. 2000, Ashby and Gupta 2013, Ryder *et al*. 2014).

Our model shows how STI prevalence – overall and in each sex – depends on both sex ratio and disease characteristics. We have shown that even when there are no sex biases in disease characteristics, increasing the transmission rate or decreasing virulence generally leads to a greater difference in STI prevalence between the sexes for a given sex ratio (Figure 4.1c-d). We have also shown that the sex ratio for which disease prevalence peaks in each sex depends on disease characteristics, again even when the disease itself shows no sex biases in transmission or virulence. The reason for these patterns can be understood in terms of a balance between an increase in the per-capita mating rate for the less common sex and a decrease in overall disease prevalence as the sex ratio becomes more skewed. Suppose the adult sex ratio is initially equal and that there are no biases in disease characteristics, hence equilibrium STI prevalence is the same in both sexes. If we

gradually remove males from the population (similarly for females), then the per-capita mating rate for the remaining males increases, and hence so does their risk of infection, at least initially. However, skewing the sex ratio in either direction reduces the overall mating rate, and therefore always lowers disease prevalence at the population level. As one continues to remove males from the population, overall disease prevalence begins to fall more sharply because uninfected males are increasingly less likely to mate with infected females. Thus, disease prevalence eventually begins to fall in males as well. Once the population crosses a critical sex ratio, R_0 falls below 1 and the disease is driven extinct. Higher transmission and lower virulence both increase overall disease prevalence and therefore buffer against the effects of skewed sex ratios, which shifts the sex ratio at which disease prevalence peaks in each sex to more extreme values. This can be seen in Figure 4.1a-b, where the gradient of the overall disease prevalence curves is flatter for higher values of β and lower values of α.

We also explored the effects of sex-biased transmission and virulence. Sex-biased disease characteristics are common in wild populations as well as in human populations (Poulin 1996, Schalk and Forbes 1997, Wilson *et al*. 2003, Lemaître *et al*. 2020), and previous studies have looked into the behavioural and physiological causes of such disparities and their implications for population dynamics (Zuk and McKean 1996, Brei and Fish 2003, Miller *et al*. 2007). However, the interaction between these biases and STI prevalence within each sex have previously been overlooked. Our model suggests that sex-biased disease characteristics may independently influence STI prevalence in a similar way as the unbiased transmission rate or virulence (see above). However, the prevalence curves for each sex are no longer symmetrical when there are biased STI characteristics (Figure 4.2). Our model also reveals that interactions between sex ratio and sex-biased disease characteristics can lead to differential and non-intuitive patterns for STI prevalence. For example, while increasing the bias in transmission from females to males causes STI prevalence in females to peak at a more extreme sex ratio, there are only marginal effects on males, yet the effects of male-biased virulence on disease prevalence are much more pronounced in males than females (as shown in Figure 4.3). The model also predicts that increasing or decreasing sex-biased disease characteristics may lead to non-monotonic variation in STI prevalence. In the absence of mathematical modelling, it is unlikely that one would be able to intuit such effects, but our results suggest that there are likely to be complex interactions between sex-biased disease characteristics and population sex ratio that shapes STI epidemiological dynamics.

Our model is simple and makes few biological assumptions. Therefore, follow-up analyses are necessary to investigate biological factors mediating STI prevalence that are not captured in our model. First, previous studies have emphasised the importance of pair formation in STI prevalence (Dietz and Hadeler 1988, Castillochavez and Huang 1995), however, our model assumes mating is random, there is no variance in mating rate, and hosts do not form pair-bonds (Knell 1999, Boots and Knell 2002, Ashby and Boots 2015, Ashby 2020). This was important for the present study as

we wanted to focus on effects of sex ratio and disease characteristics, all else being equal. However, future work would benefit from taking pair-formation into account, as a series of studies in human STIs have shown the variations in form and duration of partnerships may have an effect on pathogen prevalence (Eames and Keeling 2006, Ong *et al*. 2012, Kretzschmar and Heijne 2017, Leung *et al*. 2017). Therefore, our result might be affected due to different forms and durations of pair bond between males and female. If partnerships are monogamous, pairs of susceptible individuals are protected from becoming infected, while pairs of infected individuals delay onward transmission of the infection as long as they persist. Second, previous studies have shown how variance in mating rate (Thrall *et al*. 2000, Ashby and Gupta 2013) and mate choice (Kokko *et al*. 2002, Ashby and Boots 2015, Ashby 2020) affect disease dynamics, which in turn may influence host and STI evolution in non-intuitive ways. When one sex experiences higher variance in mating rate, the mating population is effectively sex- skewed and so we would expect this to generally have a similar effect on STI prevalence as seen in our model. If infection status impacts on mating rate (Loehle 1997, Knell 1999, Ashby and Boots 2015, Ashby 2020), then the effects may be more complex, especially if mate choice is driven by one sex.

Previous models have explored how STIs might affect the evolution of host mating dynamics (Knell 1999, Boots and Knell 2002, Ashby and Boots 2015, Ashby 2020), but the focus of the present study has been on STI prevalence. However, given that there are interactions between sex ratio, sex-biased disease characteristics and mating dynamics, there are likely to be evolutionary implications for these factors on hosts and STIs, which should be the target of future research. In particular, it is well-established that disease prevalence is important for the evolution of host defence (Haldane 1949), and so biases in sex ratio or disease characteristics are likely to influence selection for traits such as resistance, tolerance or mate choice through STI prevalence (Ashby and Boots 2015, Ashby 2020). Additionally, since females are more important than males for population growth in many species, female-biased disease prevalence might be more likely to increase population extinction risk.

Our results have several implications for STIs in natural populations. First, when sex ratios are skewed, we should expect STIs to be more prevalent in the rarer sex, although the relative prevalence of co-circulating STIs in males and females will differ due to variation in transmission and virulence (Figure 4.1c-d). For example, in a female- skewed population, an STI with a high transmission rate is likely to have a higher prevalence in males relative to females compared to an STI with a low transmission rate. Second, our model predicts that variance in the sex ratio between populations to drive STI prevalence in males and females, with divergence from equal sex ratios generally increasing sex differences in prevalence (except at extreme sex ratios). Finally, we should expect the interaction between sex ratio and sex-biased transmission or virulence to consistently have a stronger effect on STI prevalence in one sex than in the other.

4.5 Conclusion

To conclude, the objective of our model was to examine the interplay between sex ratio and disease characteristics and explore their effects on STI prevalence. Our key message is that while the less common sex is predicted to exhibit higher STI prevalence at equilibrium, disease characteristics such as transmission rate and virulence – sex-biased or not – combined with sex ratio, drive differential patterns in male and female STI prevalence.

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Competing interests

The authors declare that they have no competing interests.

Data availability

Source code is available in the Supplemental Material and in the following Github repository: https://github.com/ecoevogroup/Halimubieke_et_al_2020.

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Chapter 5 | Gastro-intestinal microbiota of two high altitude bird populations

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Manuscript

This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.

Author's Contributions

NH and TS conceived the project and designed methodology;
NH and BF carried out the statistical analyses.
NH, BF and AU led the writing of the manuscript.
All authors contributed critically to the drafts.

Abstract

Gut microbiotas contribute to shaping reproductive success, health and lifespan. Extensively studied in humans and lab animals, gut microbiotas have been increasingly explored in a wide range of taxa in the wild. Environment, sex and age influence the gut microbiota but whether this is due to intrinsic processes such as physiologic difference between sex or age groups, or knock-on effects from behaviour, for example, difference in social behaviours between sex or age groups, remains unclear. Here, we provide an insight into the composition of gut microbiota in Kentish plover (*Charadrius alexandrinus*) and lesser sand plover (*Charadrius mongolus schaeferi*) in natural populations. By analysing faecal bacterial communities using 16S rRNA sequencing technology, we find that both species have a similar and overlapping gut microbial communities with Firmicutes, Proteobacteria and Bacteroides being the dominant phyla, suggesting that similar breeding environment may be a factor shaping gut microbiota. In Kentish plovers, we found no evidence of age- and sex-related differences. Our study provides baseline information that can be used in future studies to better understand diversity, function and determinants of gut microbes in *Charadrius* shorebirds.

Keywords

Gut microbiota, 16sRNA gene, shorebirds, environment, social structure, disease biology, mating system, development

5.1 Introduction

Microbiome research is an emerging field in ecology and evolutionary biology. The gut microbiota, is a diverse community of bacteria, archaea or eukaryote that reside within the gastrointestinal tract, influencing the physiology, behaviour, and fitness of the host (Ley *et al*. 2008, Kohl 2012, Waite and Taylor 2015). Since the gut microbiota may be pivotal to important biological processes, understanding factors that shape overall gut microbiota biodiversity is of utmost interest.

Several studies have looked at the factors that shape gut microbiota, host phylogeny, environment, sex and development have been identified as important (Foster *et al*. 2017, Grond *et al*. 2018, McDonald *et al*. 2018, Watson *et al*. 2019). Gut microbial communities are more similar among conspecific individuals than among individuals of different species (Ley *et al*. 2008, Goodrich *et al*. 2014, Waite and Taylor 2014, Hird *et al*. 2015). The environment however has a significant effect on gut microbiota composition. For instance, individuals from sympatric species (e.g. migratory passerines, Lewis *et al*. 2017; chimpanzees and gorillas, Moeller *et al*. 2013) show convergent gut microbial communities reflecting their similar ecological environment and diet. In contrast, individuals from populations at different locations show divergence in gut microbiota. For example, gut bacterial diversity is significantly higher in onshore polar bears compared to offshore polar bears (Watson *et al*. 2019). Migrants consistently had higher abundances of certain bacterial genus compared to conspecific residents in sandpipers (Risely *et al*. 2018).

Gut microbiota of males and females differ in several species including humans (Elderman *et al*. 2018, Kim *et al*. 2020). Gut microbiota sex differences can result from intrinsic physiologic sex differences but may also be indirectly caused by behavioural differences between the sexes. If the former, then it could be expected that patterns of differences between males and females should be consistent across species. If the latter, gut microbiota patterns in males and females should vary according to behavioural differences in species with different social behaviours. Most studies have suggested that the sex differences in gut microbiota stem from sex-dependent physiological conditions (e.g. sex hormone and body mass index) and diet (Flores *et al*. 2012, Bolnick *et al*. 2014, Dominianni *et al*. 2015, Neuman *et al*. 2015, Org *et al*. 2016, Escallon *et al*. 2017). Another line of studies have shown that social contact can mediate the acquisition and flow of microbiomes between individuals (Degnan *et al*. 2012, Song *et al*. 2013, Nuriel-Ohayon *et al*. 2016), social contacts may allow for the horizontal gut microbiota transmission, which is presumed in direct relation to the patterns of sex-specific social contacts (e.g. social interactions, mating behaviours; Münger *et al*. 2018). For example, in wild black howler monkeys (*Alouata caraya*), gut microbiotas of adult females that generally interact more with each other showed greater similarity compared to males that have fewer social contacts with both females and other males (Tung *et al*. 2015, Amato *et al*. 2017). In lizards, the cloacal bacterial communities (cloacal bacterial samples are widely used as a proxy of gut microbiota in studies of birds and reptiles; Videvall *et al*. 2018) of polyandrous female were significantly more diverse than in monogamous females, suggesting that a larger

number of sexual partners increased bacterial diversity in females' cloaca (White *et al*. 2011). Other studies support these findings, reporting higher microbial diversity in the sex with the highest diversity of social or ecological niches (White *et al*. 2010, White *et al*. 2011, Leclaire *et al*. 2014, Levin *et al*. 2016, Amato *et al*. 2017).

Several studies have reported changes in gut microbiota along development (Cox *et al*. 2012, Faith *et al*. 2013, Grond *et al*. 2017, Videvall *et al*. 2018). For example, gut microbial communities during early life in the young are highly variable in diversity and abundance and are markedly different from gut microbiotas of conspecific adults (González-Braojos *et al*. 2012, van Dongen *et al*. 2013, Waite and Taylor 2014). However, the mechanism behind age-related gut microbial difference is poorly studied, such differences can be caused by parenting behaviour (e.g. biparental care vs. uniparental care). In several species, there is evidence of vertical transmission from mother to offspring playing an important role in the establishment of gut microbiotas during early life offspring (Nuriel-Ohayon *et al*. 2016). The extent of vertical transmission is thought to be at least partly dependent on the precocity of the young. For example, in altricial birds where young are usually dependent on their parent(s) for food, gut microbiota might be inoculated through feeding, therefore, the offspring's gut microbiota should resemble the parent(s)'. Precocial young leave the nest soon after hatch and often forage independently, thus there would be fewer opportunities for parent to offspring gut microbiota transmission.

Charadrius plovers, are a group of small ground-nesting shorebirds breeding on all continents except Antarctica (Eberhart-Phillips 2019). Kentish plover (*Charadrius alexandrinus*) and lesser sand plover (*Charadrius mongolus schaeferi*) breed at an alkaline lake in Tibetan Plateau (3200m above sea level) from May to July, and winter in coastal southern China. Both species are monogamous, males and females have roughly a similar number of close partners. They both show biparental care to the precocious offspring. They share common breeding habitat and forage mainly on aquatic insects and their larvae (fieldwork observation). They represent a good system to test the influence of environmental and social behaviour in gut microbiota.

Here, we analyse microbial biodiversity in faecal samples to assess gut microbial community female, male and young individuals in wild populations of Kentish and lesser sand plover to assess relative relevance of genetic differences between species, social links or environment in shaping the gut microbiota in these two species. Here we explore three hypotheses that assume distinct major drivers of gut microbiota diversity and composition at different scales. First, if the gut microbiota composition is mainly determined by underlying genetics, we would expect significant differences in microbial communities between the two species analysed. However, if environment conditions are the main force to shaping gut microbiota composition, we expect gut microbiota composition to be similar in both species as both inhabit the same environment during their breeding season. Second, if physiological differences between the sexes influence gut microbiota, then we expect male and female microbiomes to be different. However,

if sex differences are explained by differences in social networks, home ranges and foraging behaviour, then we expect that microbiomes in these monogamous and biparental caring birds to be similar for males and females. Third, if differences in gut microbiota between adults and juveniles are explained by intrinsic differences in the physiology of the two life stages, then we would expect to observe differences in the two species included in this study. However, if these differences are the result of the differences in diet of adults and young (i.e. in mammals, young are fed mother's milk and in birds, many young are provided with food by parent(s)), we would expect that gut microbiota composition should not differ between adults and juveniles in these two precocial species where young fetch their food by themselves.

5.2 Materials and Methods

5.2.1 Sample collection

Fieldwork was carried out at Qinghai Lake, an alkaline lake lies in Tibetan Plateau, in May and June 2019. Kentish plovers and lesser sand plovers breed along the lake shore. Breeding pairs were captured on their nest while incubating eggs, using funnel traps (Székely *et al*. 2008). The sex of adult birds was determined by morphological characters, and molecular sexing was applied to identify the sex of the chicks. Gut microbiota samples were collected from captured adults and chicks. Faecal samples are generally representative of the bacterial community in the large intestines. We adopted Knutie and Gotanda's (2018) faecal sampling protocol. The captured bird was put into a paper bag with a sterile wax paper on the bottom. A metal grate was set over the wax paper to prevent the bird from directly encountering the faeces after defecation. After defecation, faecal samples were collected using sterile polyester swabs, placed in sterile cryotubes without medium, and kept in liquid nitrogen. The grate was sterilized by soaking them in a 10% bleach solution for at least 10 min before each collection to reduce potential cross-contamination between different individuals. The bird was held for less than 5 min to minimize stress and then be released. Samples were transported from the liquid nitrogen to a laboratory freezer at -80 °C. As studies suggest differences in bacterial composition resulting from storage conditions do not eclipse differences between samples, even when left at ambient temperatures for 2 weeks (Lauber *et al*. 2010, Dominianni *et al*. 2014, Song *et al*. 2016), we assume that changing of the storage environment had minimal effect on microbial composition of the samples collected.

5.2.2 DNA isolation, amplification and sequencing

To obtain sufficient amounts of genomic DNA for sequencing library preparation, the faecal sample on the sterile tips were used to extract genomic DNA with the FastDNA Spin Kit for Faeces (MP Biomedicals Co., Ltd., USA) following the manufacturer's instructions. Then, the concentration and purity of the extracted DNA samples were measured using a Nanodrop 2000 spectrometer (Thermo Fisher Scientific, Wilmington, DE, United States). Bacterial diversity was examined after

250-bp paired-end amplicon sequencing using the primers 515F (5-GTGYCAGCMGCCGCGGTAA-3) and 806R (5-GGACTACNVGGGTWTCTAAT-3) on an Illumina Miseq platform in two separate runs at Suzhou GENEWIZ, Co., Ltd, (Suzhou, China).

Paired-end reads were merged, and pairs diverging by more than 200 bp or containing unknown base calls (N) were discarded. Then the PCR amplification primers were trimmed, and the sequences were quality filtered at 0.5% Expected Error (EE); those displaying greater than 0.5% EE were discarded. Using the VSEARCH (ver. 1.9.6) algorithm, operational taxonomic units (OTUs) were created by clustering sequences with 97% sequence identity, discarding chimeric sequences after being aligned to the SILVA reference. Taxonomic assignments of representative sequences from each OTU were performed using the Ribosomal Database Program (RDP) Bayesian algorithm. Sequences with ≥ 97% similarity were assigned to the same OTU.

5.2.3 Statistical analyses

All the faecal samples were classified into three groups: (a) species group (Kentish plover vs. lesser sand plover); (b) sex group (female Kentish plover vs. male Kentish plover); (c) age group (adult Kentish plover vs. juvenile Kentish plover). We analysed bacterial communities in three ways. (1) To identify the OTUs that differ in abundance within each comparison group, we applied the Metastat gap analysis. (2) To examine community-wide differences in abundance within each comparison group, we fitted non-multidimensional scaling (NMDS, vegan package in R; R Core Team 2018) ordinations to rarefied count data, the results were presented based on Bray-Curtis (based on abundance of OTUs), then we conducted analysis of similarities tests (ANOSIM, vegan package in R; R Core Team 2018) to statistically test for differences within each group. (3) We analysed community diversity by calculating Alpha diversity indices (chao, Shannon and Simpson) using Qiime (ver. 1.9.1) and the t-test or Wilcoxon test was used to compare the differences within each comparison group.

5.3 Results

A total of 5254 OTUs were identified from cloacal samples of 24 Kentish plovers (17 adults and 7 juveniles) and 9 lesser sand plovers (7 adults and 2 juveniles) encompassing 36 bacterial phyla, with prevalence and abundance of specific phyla differing among individuals (Figure 5.1a). The dominant phylum in both species show consistency, with the most abundant phyla being Firmicutes (60% in Kentish plovers, 59% in lesser sand plovers), Proteobacteria (14% in Kentish plovers, 15% in lesser sand plovers) and Bacteroidetes (10% in Kentish plovers, 14% in lesser sand plovers), making up the core microbiota (Figure 5.1b).

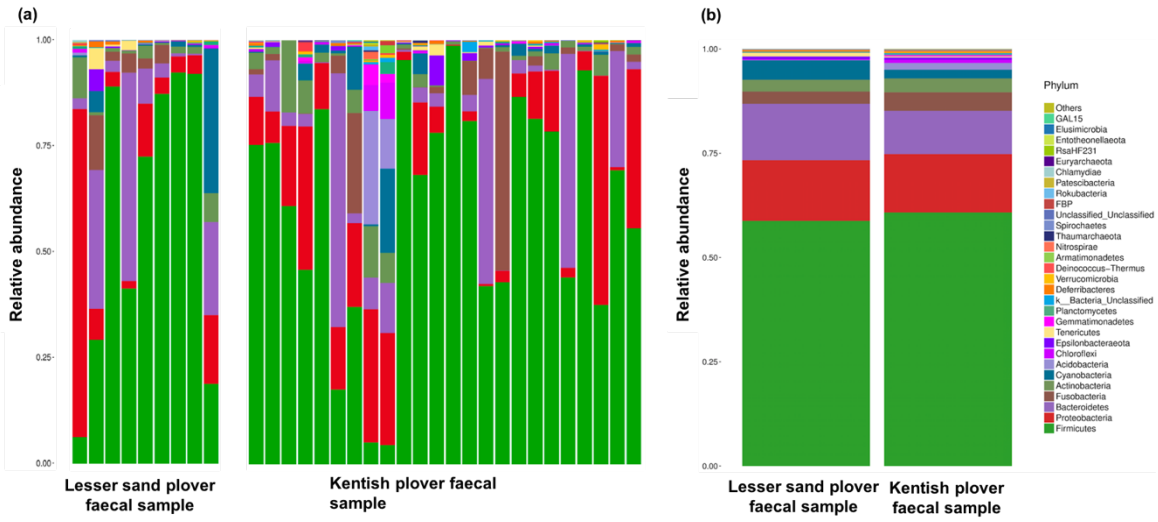

Figure 5.1 (a) Stacked bar chart of the relative abundance of top 30 bacterial phyla in the faecal microbiota of 24 Kentish plovers and 9 lesser sand plovers. **(b)** Stacked bar chart of the average relative abundance of bacterial phyla in faecal microbiota of Kentish plovers and lesser sand plovers. Phyla in the legend are listed in order of increasing abundance.

5.3.1 Differences in bacteria taxa

Kentish plover vs. lesser sand plover

The faecal microbiota of Kentish plover consisted of 3848 OTUs compared to 1406 OTUs for lesser sand plovers, of which 1208 were shared between two populations (Figure 5.2a). Of the total number of OTUs found, 2640 were unique to Kentish plovers, and a much smaller number of OTUs (n = 198) were unique to lesser sand plovers.

5 OTUs (2 Bacteroidetes; 2 Acidobacteria; 1 Firmicutes; 1 Proteobacteria) were significantly enriched and 1 OTU (1 Proteobacteria) was significantly reduced in Kentish plover faecal samples (Figure 5.3a). We also found that the relative abundance of bacteria genus of Corynebacterium_1, Euzebya and Ruminococcaceae_UCG-008 showed significant difference between Kentish plovers and lesser sand plovers (Figure 5.4a-c, $P_{\text{Corynebacterium\_1}} = 0.04$, $P_{\text{Euzebya}} = 0.02$, $P_{\text{Ruminococcaceae\_UGC-008}} < 0.01$; Metastats).

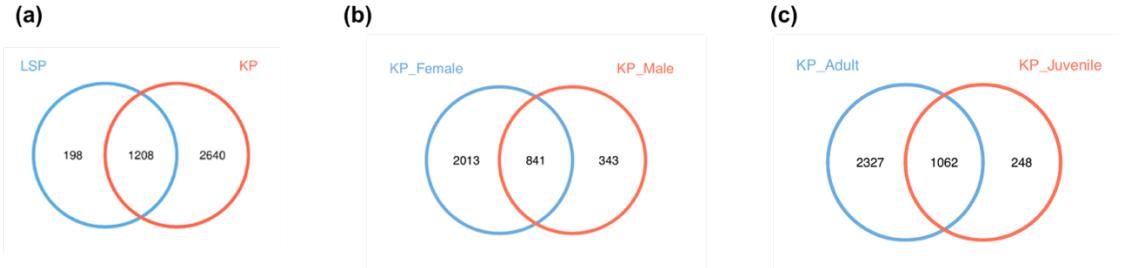

Figure 5.2 Venn diagrams demonstrating number of shared and unique OTUs in **(a)** Kentish plovers (KP, red) and lesser sand plovers (LSP, blue), **(b)** in female (blue) and male (red) Kentish plover, and **(c)** in adult (blue) and juvenile (red) Kentish plovers.

Female Kentish plover vs. male Kentish plover

Female Kentish plover faecal microbiota consisted of 2854 OTUs, and the number in male Kentish plovers is 1184 OTUs. 841 OTUs were shared between sexes (Figure 5.2b). A total 2013 OTUs were found unique to females, and 343 to males.

9 OTUs (4 Proteobacteria; 3 Firmicutes; 1 Gemmatimonadetes; 1 Actinobacteria) were found more abundant in female Kentish plovers and 1 OUT (Firmicutes) in males (Figure 5.3b). At a finer scale, we found that the relative abundance of bacteria genus of Acinetobacter showed significant difference between female and male Kentish plovers (Figure 5.4d, $P_{\text{Acinetobacter}} = 0.01$).

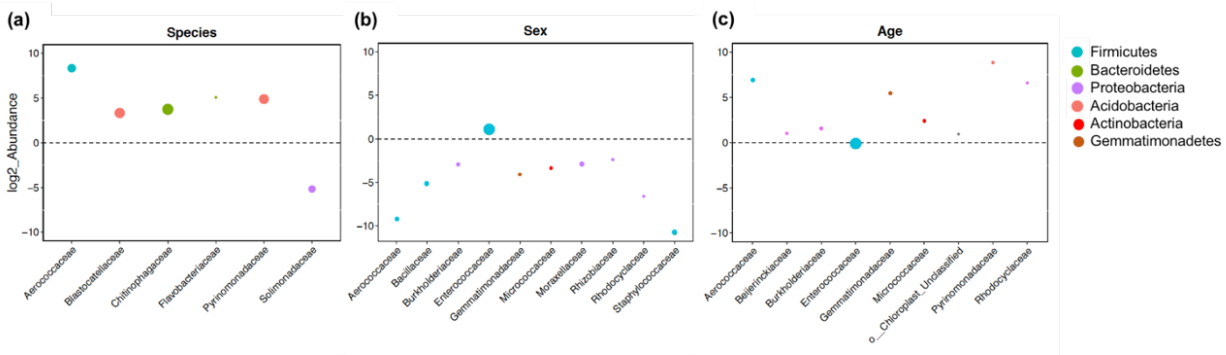

Figure 5.3 Fold changes for OTUs (circles) that significantly differed between **(a)** Kentish plovers and lesser sand plovers, **(b)** female and male Kentish plovers and **(c)** adult and juvenile Kentish plovers. OTUs below the dashed line are more abundant in lesser sand plovers in (a), female Kentish plovers in (b) and juvenile Kentish plovers in (c). OTUs are grouped by family, coloured by phyla, and sized by mean relative abundance across samples.

Adult Kentish plover vs. juvenile Kentish plover

The number of OTUs in adult Kentish plover faecal microbiota is 3389, and the number in juvenile Kentish plovers is 1310. 1062 OTUs were shared between adults and juveniles (Figure 5.2c). 2327 OTUs were unique to adults compare to 248 for juveniles.

7 OTUs (3 Proteobacteria; 1 Firmicutes; 1 Gemmatimonadetes; 1 Actinobacteria; 1 Acidobateria) were found more abundant in adult Kentish plovers and 1 OUT (Firmicutes) in juveniles (Figure 5.3c). From genus level, the relative abundance of the genera of Butyricicoccus, Exiguobacterium, Lactococcus and Serratia showed significant difference between adult and juvenile Kentish plovers (Figure 5.4e-g, $P_{\text{Butyricicoccus}} = 0.02$, $P_{\text{Exiguobacterium}} = 0.02$, $P_{\text{Serratia}} = 0.03$).

5.3.2 Differences in community-wide abundance and species diversity

Our results showed that Beta diversity showed no significant difference between each comparison group when using Bray-Curtis (Species: $R = 0.14$, $P = 0.10$; Sex: $R = -0.01$, $P = 0.47$; Age: $R = -0.05$, $P = 0.62$; ANOSIM).

Alpha diversity did not differ between each comparison group, either. For between species: Chao index (P = 0.37, Wilcoxon test), Shannon index (t = -0.04, df = 31, P = 0.97, t-test) and Simpson index (t = -0.44, df = 31 P = 0.66, t-test). For between sexes: Chao index (P = 0.67, Wilcoxon test), Shannon index (t = 1.79, df = 22, P =0.09, t-test) and Simpson index (t = 0.94, df = 22, P = 0.36, t-test). For between age groups, Chao index (P = 0.66, Wilcoxon test), Shannon index (t = 0.66, df = 22, P = 0.51, t-test) and Simpson index (t = 0.12, df = 22, P = 0.90, t-test).

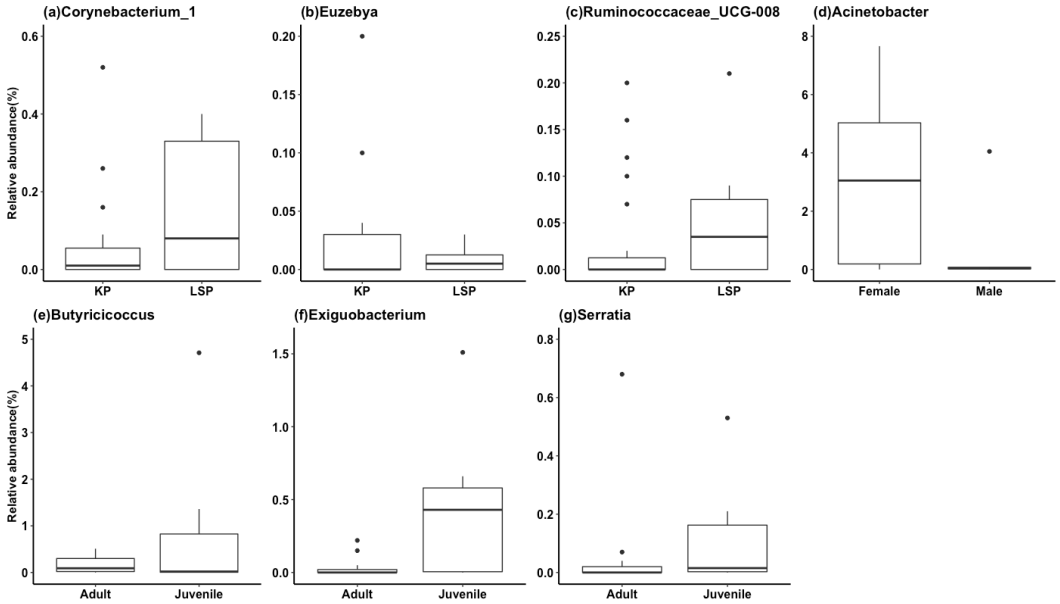

Figure 5.4 Relative abundance of bacterial genus in the faecal microbiota between Kentish plovers and lesser sand plovers (**a-c**), female and male Kentish plovers (**d**), and adult and juvenile Kentish plovers (**e-g**).

5.4 Discussion

In this staged study, due to the limited knowledge in *Charadrius* plover gut microbiota, we first investigated the gut microbiota composition in two sympatric *Charadrius* plover populations that breed in high altitude environment. We found that at the phylum level, the most abundant phyla in Kentish plover and lesser sand plover faeces are Firmicutes, Proteobacteria and Bacteroidetes, accounting for nearly 90% of the total gut microbiota. This result was consistent with previous studies of the gut microbiota in other avian species (Roggenbuck *et al*. 2014, Hird *et al*. 2015, Grond *et al*. 2018). Firmicutes are associated with mass gain and immune function in mammals and birds, playing an important role in increasing nutrient uptake and metabolic efficiency (Flint *et al*. 2012, Zhang *et al*. 2015, John and Mullin 2016), whereas Bacteroidetes is proposed to play a specific role in break-down of cellulose and other plant materials (Thomas *et al*. 2011, Kohl *et al*. 2014). However, the function of Proteobacteria in birds remains undetermined. It is also noteworthy that considerable number of Fusobacteria was seen in Kentish plover (relative abundance 4.4%)

and lesser sand plover (relative abundance 2.9%) faecal samples. Fusobacteria are often studied in the context of pathogenicity, and in carnivorous birds, Fusobacteria seem to be beneficial for the resistance against pathogens (Roggenbuck *et al*. 2014, Mendoza *et al*. 2018). Therefore, it is an interesting avenue for future study to investigate the occurrence of Fusobacteria in shorebirds.

Although the abundance of the dominant phyla did not differ significantly in any of the comparison groups, some bacterial families and genera differ in abundance across comparison groups (Figure 5.3 and Figure 5.4). At family level, we noticed that the Aerococcaceae family shows consistent biased across the comparison groups, however, we found that this is because one Kentish plover (adult, female) sample contained extremely high level of Aerococcaceae (8.5%), whereas the numbers in the other samples were lower than 0.03%, which skewed the results. Similar patterns were found among Burkholderiaceae distribution in age and sex groups. There are several reasons to explain such sharp variation in abundance: first, it is probably due to sample contamination or the sample size was too small to explain the evident abundance variations in some bacterial taxa. Second, the 97% divergence of 16s sequencing probably is too broad to miss many finer variations that may be important. At the genus level, we found that Corynebacterium and Ruminococcaceae were more abundant in lesser sand plover than in Kentish plover (Figure 5.4a-b). Corynebacterium genus is believed to be more abundant in migratory than conspecific residents in *Calidris* shorebirds, it may enable migrating shorebirds to maximise fat deposition and/or energy harvest during migration (Risely *et al*. 2018), whereas the abundance of Ruminococcaceae has been linked with the maintenance of gut health (Zheng *et al*. 2019). Therefore, it is possible that life history traits, such as migration, and the health status of individuals may also account for the different abundance in some bacterial genus. However, the function roles of much of the OTUs in this study is still poorly known.

Our results also showed that within each comparison group, Alpha diversity and Beta diversity showed no difference, suggesting individuals from each comparison group had similar and overlapping gut microbial communities. First, gut microbial composition did not significantly differ between Kentish plovers and lesser sand plovers, suggesting that gut microbiota composition is probably influenced more by environmental factors than genetic factors. We posit that similarity in gut microbiota composition between Kentish plovers and lesser sand plover may stem from the similar range of diets and habitats experienced by both species prior to sampling (David *et al*. 2014, Hird *et al*. 2014, Carmody *et al*. 2015). However, our proposition needs to be tested by further research so as to better understand the role of environment and genetic factor in shaping gut microbiota. For example, to compare the gut microbiotas within same species but from different ambient environments (e.g. migrotary vs. conspefic resident; Risely *et al*. 2018). Furthermore, Ley *et al*. (2018) suggest that gut microbial communities are more similar among closely related species, as Kentish plover and lesser sand plover populations in this study are not only sympatric, but also closely related congeners. Therefore, another possible aspect for future research is to involve more

species that are less genetically related to each other but share common environment. In this study, it would be beneficial to compare gut microbiota of Kentish plovers and lesser sand plovers with other sympatric avian species (e.g. geese, passerines).

Second, within Kentish plovers, sex seems to show no influence on gut microbiota. Studies of human and other mammalian species show that males and females differ in reproductive physiology and behaviour, which may manifest as different gut microbial profiles (Neuman *et al*. 2015, Aivelo *et al*. 2016, Grond *et al*. 2018). However, there were no significant sex-related differences in gut microbiota in Kentish plovers. The homogeneity in gut microbiota suggests that the influence of fundamental biological difference between sexes on gut microbiota may be obscured by other factors, such as social behaviour and ambient environment. However, we are not yet able to determine the roles of social behaviour and ambient environment in shaping the sex-specific gut microbiota, given that the Kentish plover population in this study is monogamous, indicating a similar social behavioural pattern between males and females. Therefore, future study would benefit from a comparative result from a population with sex difference in social behaviour, for example, a population with females being polygamous, and males being monogamous. In addition, a study shows that mating behaviours could cause transient change in gut microbiota in black-legged kittiwakes *Rissa tridactyla* (White *et al*. 2010), indicating that the time of sampling might also influence the gut microbiota (e.g. pre-mating vs. post-mating), therefore, repeated sampling is required in future work to better understand the sex-difference in gut microbiota.

Third, we did not see any age-related differences in gut microbiota in Kentish plovers either, although studies investigating microbiota in precocial or altricial birds showed that the gut microbiota composition of chicks is markedly different from that of adult birds (González-Braojos *et al*. 2012, Grond *et al*. 2017). However, it is worth noting that chick samples are richer in some bacterial genus than adults, for example, Exiguobacterium and Serratia (Figure 5.4f-g). Most Exiguobacterium and Serratia are non-pathogenic environmental bacteria, with the former has ability to survive in varying temperature extremes (Vishnivetskaya *et al*. 2009, Khanna *et al*. 2013). This suggests that although the community-wide abundance and species diversity show no difference between adults and juveniles in this study, the development stage of juveniles should be an important factor to be accounted for, since the microbial colonisation in shorebird chicks starts soon after hatching, and changes throughout development (Grond *et al*. 2017). In addition, as is mentioned above, limited sample size and sample contaminations may also conceal the real pattern of gut microbiota between sexes and age groups.

In summary, this study described and compared the gut microbiota of Kentish plovers and lesser sand plovers. The preliminary results showed that the similar breeding environment may have caused homogeneous gut microbiota patterns between the species. Within Kentish plover, gut microbial diversity showed no difference between sex or age group. This study also provides baseline information and methodologies that can be used in future studies to better understand the

broad-scale patterns in gut microbiota and function in wild birds and assessed how gut microbiota of shorebirds relates to genetic, environmental factors, population social structure, immune function, and disease-related processes.

However, given this study is a phased study, there are several major limitations that are concluded as follow, which can be references for future study: First of all, due to the time constraint, we did not collect samples from populations with contrasting breeding systems and surrounding environments. Therefore, for future study, we need to expand the research scale by including more species and populations with contrasting social behaviours from a wide geographic range. This will improve our very limited understanding of gut microbiota in wild birds (especially shorebirds). Second, as is mentioned above, gut microbiota composition is a dynamic process which might be influenced by factors such as sampling time, sampling repetition, genetic and social relationship between individuals (White *et al*. 2010; Grond *et al*. 2017). However, in this study, we did not control for these important factors, which might have led to the high variations in gut microbiota patterns between individuals. Therefore, except for broadening sampling, repeated sampling, social behaviour monitoring and genetic pedigree building are essential for us to better assess different aspects that influence gut microbiota of birds under natural conditions. Third, the OTUs were created by clustering sequences with 97% sequence identity in this study, which is a "broad brush" taxonomy that may omit a lot of finer variation between individuals. In addition, our understanding of the functional roles of much of gut microbial communities in these populations is scarce, more advanced and sensitive sequencing techniques should be applied. The application of shotgun metagenomics, metatranscriptomics and metaproteomics on elucidating the functional potential of gut microbial communities in previous studies would offer valuable insights for avian gut microbe dynamics in future research (Pérez-Cobas *et al*., 2013; Kato *et al*., 2014). Finally, the pathogenic functional profiles of gut microbial community are essential for our understanding of the link between social structure and disease prevalence and has significant implications in conservation biology.

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Ethical statement

This study did not involve any manipulation experiments, and all methods were carried out in accordance with relevant guidelines and regulations of the National Forestry and Grassland Administration, PRC. Birds were ringed and handled by trained people aiming to cause as little disturbance to birds as possible.

Conflict of interest

The authors declare that they have no competing interests.

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Chapter 6 | Discussion

Understanding animal social behaviours, especially breeding behaviours, has been a central topic of behavioural ecology for over 50 years (Wilson 1975, Trivers 1985, Székely *et al*. 2010, Davies *et al*. 2012, Rubenstein and Alcock 2018). Over the years, there have been many attempts to understand the theoretical, experimental and phylogenetic aspects of those immensely diverse behaviours (Székely *et al*. 2010, Westneat and Charles 2010, Royle *et al*. 2012). The main objectives of my dissertation are to understand how mating decisions are made by individuals and how social structure and demography are related to the prevalence of disease infections in wild populations. In order to address these questions, I focus on *Charadrius* plovers – a group of shorebird species that exhibit diverse breeding systems – in empirical and theoretical approaches. My dissertation goes beyond previous studies of breeding systems in three respects:

(i) Previous studies and hypotheses of mate fidelity mostly centre around socially monogamous birds that exhibit long-term pair bonds (Black 1996, Dubois and Cezilly 2002, Bried *et al*. 2003). However, these works have not explained why pairs of some species show variable pair-bonds (e.g. sequential polygamy) within breeding years. My work integrates long-term monitoring data from 14 plover populations across the globe, and for the first time comprehensively quantifies mate fidelity of plover individuals and investigates the ecological factors and life-history traits that are related to individual mating decision processes.

(ii) Using a theoretical model, I explore for the first time how population sex ratio and disease characteristics cause differences in STI prevalence between the sexes.

(iii) Finally, my experimental work using bioinformatics explores the gut microbiota in *Charadrius* plovers and provides an exciting avenue of future empirical research of social structure and disease biology in wild populations.

My dissertation provides three main results: (i) successful breeding leads to divorce, whereas failed breeding leads to retention of the mate for follow-up breeding in plovers (Chapters 2 & 3); (ii) in a population with a biased sex ratio, the less common sex exhibits higher STI prevalence. Disease characteristics (i.e. transmission rate and virulence), sex-biased or not, combined with sex ratio, together shape the STI transmission dynamics (Chapter 4); (iii) I provide an insight into the composition of the gut microbiota in two high altitude plover populations and preliminary results reveal no relation between gut microbiota composition and social structure (i.e. age and sex; Chapter 5). Below I discuss each of these major findings.

6.1 Breeding success and mate fidelity

Evolutionary theory predicts that individuals make different mating decisions in order to maximise reproductive success (Houston and McNamara 1999, McNamara and Weissing 2010). The key to understanding why individuals make different mating decisions lies in the costs and benefits of mating decisions. Evidence across a wide range of taxa suggests that retaining a mate for future reproduction is beneficial (Griffith *et al*. 2002, Whiteman and Côté 2004, Lukas and Clutton-Brock 2013), because mate retention can improve reproductive success by reducing time and energy cost of searching for a new mate (Adkins-Regan and Tomaszycki 2007, Perfito *et al*. 2007), or by improving breeding performance of the pair (Ens *et al*. 1996, Gabriel and Black 2013). However, in my study, I found that plovers benefit from divorce instead of mate fidelity, individuals that divorced their partners and simultaneously deserted their broods produced more offspring than parents that retained their mate. What is behind those contradictory patterns across taxa? I posit that mating decision for future reproduction are the result of various constraints related to life history traits, social and ecological environmental factors among populations, and the relative importance of these factors most likely varies among animal taxa.

Life history traits and ecological conditions can have an important influence on mating decision via setting different kinds of "time constraints". Life history traits, such as longevity, are expected to constrain the length of the reproductive life span of an individual thus is related to different mating decisions. For example, empirical evidence has shown that divorce is mainly triggered by reproductive failure in long-lived socially monogamous species (Ens *et al*. 1996, Culina *et al*. 2014), whereas in short-lived species like butterflies, given time-consuming copulations, adults cannot afford multiple breeding attempts (Wiklund and Forsberg 1991). Under these conditions, divorce rates should be higher in long-lived species than in short-lived ones (McNamara and Forslund 1996).

Ecological conditions can also affect mating decision via setting ecological time constraints. In a wide range of taxa, environmental conditions determine the length of suitable breeding periods, thus populations in harsh and unpredictable environmental conditions often have a short time to undertake multiple breeding attempts (Gilburn and Day 1994, Welch 2003, Malpaux 2006, Välimäki and Kaitala 2006, Saalfeld and Lanctot 2015, Eberhart-Phillips 2019). Plovers are seasonal breeders so there are always time constraints in a breeding season, especially populations from high latitude where the breeding season is shorter. In addition, plovers in general suffer from high nest mortality (Kubelka *et al*. 2018, Méndez *et al*. 2018). Given that searching and courting a new mate is time consuming, re-nesting with the current mate after nest loss is an optimal strategy to make up for the time constraints. It is worth mentioning that, compared to insects and mammalian species, where breeding failure is related to partner compatibility (de Crespigny *et al*. 2008, Schwensow *et al*. 2008), predation seems to be the major driver of breeding failure in plovers,

therefore, re-mating seems more important than changing partners and risking not finding a new mate.

Parental care and ASR can also influence mating decisions via altering the mating opportunity within a population. Uniparental care is suggested to affect mate availability as the non-caring sex often returns to the mating pool after giving up the parental duty (Queller 1997, Kokko and Jennions 2008). Studies in insects and birds show that parental care may relate to ecological conditions. For example, harsh environments, ephemeral food sources or predation pressure usually call for biparental care, whereas uniparental care is favoured in mild ecological conditions, because with adequate resources, one parent is able to rear the offspring (Alonzo and Hope 2012, Wong *et al*. 2013, Vincze *et al*. 2017). In many birds and reptiles, parental care is also related to the precocity of the offspring, because precocial offspring require minimum care, therefore one parent can be released from parental duty and become sexually available (Gill 1995).

A biased ASR is related to various sex-specific processes at various life cycle stages (e.g. a biased sex ratio at birth, sex-differential mortalities of young and adults, sex-differential maturation times, and sex-differential dispersal and migration patterns; Bessa-Gomes *et al*. 2004, Veran and Beissinger 2009, Lee *et al*. 2011, Székely *et al*. 2014). According to mating market theory (Schacht *et al*. 2017), populations with female-biased ASRs should have higher mating opportunities for males so that males may mate polygamously. In contrast, populations with male-biased ASR would benefit the females so that they should exhibit female polygamy (Székely 2019). This is supported by several lines of research in frogs, birds and mammals (Alho *et al*. 2010, Liker *et al*. 2013, Kappeler 2017). In my study, plovers show male-biased ASR and the chicks are precocial (Székely *et al*. 1999, Eberhart-Phillips *et al*. 2017, Eberhart-Phillips *et al*. 2018). The mating opportunity is usually in females' favour, thus, the best strategy to improve number of offspring is to leave the current brood to the male and re-mate with a new mate.

To sum up, the studies of mating decision-making processes in plovers provided a counterpoint to theoretical expectations that divorce is triggered by low reproductive success. On the contrary, the mating strategy of brood desertion and polygamy leads to short-term fitness, as individuals sire more offspring than those who did not divorce. Notably, life-history traits, alongside with the social and ecological environments, appears to play a strong role in shaping mating decisions in plovers. Furthermore, harsh ecological environments impose time-constraints in breeding seasons, thus restricting the opportunity for multiple breeding attempts. However, one major limitation in these two closely related studies is that the patterns between mating decisions and other predictors are correlational, to better understand the cause and effect among these components will require more experimental studies.

6.2 ASR, disease characteristics and disease prevalence

ASR not only plays an important role in shaping individual mating decisions, but also relates to the spread of infectious disease, which leads to the second major insight of my research. I found that ASR and disease characteristics cause differences in STI prevalence between the sexes. ASR can influence the transmission dynamic of STIs by altering mating opportunities among individuals (Chapter 4). For example, in a population with biased ASR, the less common sex often has more prospective mates and a higher mating opportunity than the more common sex. With high variance in mating success, many individuals of the more common sex may remain unmated or have a low mating rate, whereas the less common sex has a higher mating rate and hence greater exposure to infectious partners. Thus, disease prevalence in the more common sex is expected to be lower than in the rarer sex. This finding is consistent with previous theoretical and empirical studies in human and non-human societies (Thrall *et al*. 2000, Ashby and Gupta 2013, Bien *et al*. 2013, Ryder *et al*. 2014).

However, previous studies have overwhelmingly focused on investigating the relationship between host mating system structure and STI dynamics (Thrall *et al*. 1997, Kokko *et al*. 2002, Ashby and Gupta 2013), whereas the effects of ASR and other social demographic factors are either ignored or assumed to be fixed in a population. In reality, variation and stochasticity in ecological and social environments (e.g. weather condition, predation etc.) often skew the population ASR conditions, as well as change individual behaviours and physiological condition, which potentially lead to sex-biased traits in transmission and virulence of the STIs (Sheridan *et al*. 2000, Miller *et al*. 2007). The interactions between ASR and disease characteristics may lead to a complicated transmission dynamic of disease in wild populations. My study, to my knowledge, is the first to take disease characteristics into account, and reveal an interaction between sex ratio and sex-biased disease characteristics to consistently have a strong effect on STI prevalence.

Based on these novel results, further questions remain unsolved in this theoretical work. First, my theoretical model assumes hosts do not form pair-bonds and that mating is random among individuals, however, random mating is not common in wild populations. Individuals may show pathogen avoidance during mate choice, and pair-bonds may vary in terms of form and duration, which might complicate the transmission dynamics (Tybur and Gangestad 2011). Second, some infections may cause sterility among individuals and thus impact the mating dynamics within a population (Ashby and Boots 2015, Ashby 2020). Third, there is often a trade-off between transmission rate and virulence (Leggett *et al*. 2017), and they both interact with host immune function (Lipsitch and Moxon 1997). Therefore, future works should account for those factors to improve the generality of the theoretical model. It is also noteworthy that studies understanding the relationships and interactions between ASR, mating system and STI transmission provide an interesting avenue for future work. As social structure covers a wider range of social behavioural

traits including breeding behaviours, therefore, it would be interesting to test how the transmission dynamics of infectious diseases (STIs and non-STIs) relate to different aspects of social behaviours.

6.3 Gut microbiota

The study of gut microbiota is an important component in my research framework because of its relationship with social structure and disease biology (Azambuja *et al*. 2005, Tsiodras *et al*. 2008, Roggenbuck *et al*. 2014). Previous works have investigated the gut microbiota of many species in term of composition and biological function (Kostic *et al*. 2013, Grond *et al*. 2018). Another line of study also sheds light on how host phylogeny, environmental exposures and life history traits shape the gut microbiota across human and non-human species (Foster *et al*. 2017, Grond *et al*. 2018, McDonald *et al*. 2018, Watson *et al*. 2019). However, the studies of gut microbiota and social structure is drawing a lot of attention, because social contact has been proven to be a mechanism that can mediate the acquisition and flow of microbiomes among individuals (Degnan *et al*. 2012, Song *et al*. 2013, Nuriel-Ohayon *et al*. 2016).

Although shorebirds demonstrate complicated social behaviours and are ideal models for investigating gut microbiota and social structure, little is known about gut microbiota in shorebirds (Grond *et al*. 2018), especially *Charadrius* plovers. Therefore, I started with an investigation on plover gut microbiota composition (Chapter 5). Consistent with previous studies of gut microbiota, the dominating bacterial phyla in plover gut microbiota resemble mammals and other birds (Waite and Taylor 2014). Several lines of research suggest that the composition of gut microbiota of vertebrates is influenced by various environmental and social factors (reviewed by Grond *et al*. 2018). For example, in birds and mammals, differences in gut microbiota composition have been found between conspecific populations residing in different environments (Degnan *et al*. 2012, Risely *et al*. 2018, Watson *et al*. 2019). Studies also show that the differences in social behaviour between males and females, adults and infants may result in different patterns of gut microbiota in different species (reviewed in Chapter 5). In my study, the preliminary results show the two sympatric plover populations have similar and overlapping gut microbial communities, suggesting that the similar diet and habitat are likely the key influencing factors in the diversity and abundance of gut microbiota. This study, however, failed to prove sex- or age-related differences in gut microbiota in Kentish plover.

The investigation of gut microbiota in these two populations is a phased study, there are several reasons to explain the findings. First, in this study, data was collected from only one breeding area due to the time constraints, thus, to clarify the role of ambient environment in shaping gut microbiota composition, a larger sample size from populations with ecological settings is required. Second, the Kentish plover population used in this study is monogamous and the social behavioural patterns are similar between males and females. Comparison with polygamous populations may therefore help better understand the relationship between social behaviours and

gut microbiota. Third, microbial colonisation in shorebird chicks has been proven to start soon after hatching and changes throughout development (Grond *et al*. 2017). Repeated sampling in chicks is therefore essential to understand how age influences gut microbiota. Finally, the field sampling methods need to be improved so as to reduce contamination.

For the next stage, it would be interesting to look into the relationship between gut microbiota and social structure. Considerable amount of studies across taxa have suggested a role of social interaction in shaping an individual's gut microbiota. For example, studies in non-human primates suggest that the strength of social interactions between individuals contributes substantially to shaping the individuals gut microbiota (Tung *et al*. 2015, Moeller *et al*. 2016, Amato *et al*. 2017). In addition, a series of studies across taxa indicate that the more socially active individuals often register a larger microbial diversity (White *et al*. 2010, White *et al*. 2011, Leclaire *et al*. 2014, Levin *et al*. 2016, Amato *et al*. 2017). However, the relevant study in birds is relatively inadequate. Investigating gut microbiota in relation to social structure in birds is promising, because (i) given the reproductive anatomy, studies in birds can provide interesting angles on whether gut microbiota is transmissible via sexual interactions (White *et al*. 2010); (ii) social behaviours are highly variable in birds making them ideal models to investigate gut microbiota and different aspects of social structures; (iii) gut microbiota of birds may function as a reservoir for potential pathogens, therefore, gut microbiota is particularly significant in understanding STIs in birds (Azambuja *et al*. 2005, Tsiodras *et al*. 2008).

6.4 Future directions

The study of animal social behaviour has been fruitful in many areas of evolutionary biology and physiology behavioural ecology, wildlife biology, conservation biology, animal welfare and bio-medical sciences. My PhD work makes contributions to the understanding of the interactions among life history traits, ecological/social environments and mating decision processes and their implication in disease biology. However, further studies are needed to uncover various aspects of social behaviours and their implications in a series of key issues in evolutionary ecology and wildlife biology. In order to make further progress in this field I suggest pursuing three major avenues:

1. In my dissertation, the massive amount of field data has allowed me to understand how ecology, social environment and life history are related to the mating decision processes in wild plover populations. However, much as the patterns are interesting, they show only a correlational relationship, the cause and effect between these components and mating decision remain to be tested. Therefore, experimental studies are required for future work. In previous experimental works on plover populations (Székely *et al*. 1999, Parra *et al*. 2014), the authors broke pair-bonds by removing one of the mates and holding them in captivity. When the captive mates were released, populations responded differently. In

population with biparental care, individuals tended to re-establish pair bonds with their original mate, whereas in populations with uniparental care, the pair-bond broke. These two studies provide evidence that mate fidelity is tightly linked to parental care strategies. On the basis of these two studies, we can advance this research field by carrying out similar experiments, but using a comparative approach. One plausible and feasible research plan is to focus on the plover populations in China, because (i) our research group has established long-term field monitoring on five plover populations in China over the past years; (ii) these five populations vary greatly in ecological settings, ranging from coast to plateau, from tropic to desert; (iii) the five populations are closely related, yet show variations in breeding system, some are monogamous showing biparental care, some are polygamous with uniparental care; (iv) most importantly, we have built in-depth collaborations with local institutes, which will provide efficient preparation and coordination for this experimental study. By carrying out similar mate-removal experiment among these five populations, we can investigate how ambient environment, life history traits and social environment predict the mating decisions.

2. Another important avenue would be to explore the relationship between population social structure and disease-related processes using theoretical and empirical approaches. This requires two lines of research. First, now that my research has provided a theoretical support for linking ASR and STIs, further research is needed to improve the theoretical model by including more biological factors (e.g. pair-bond, parental care) that mediate disease prevalence. The second line of research requires empirical studies from three aspects to support the theoretical model: (i) recent studies have demonstrated the implication of population social structure in mating behaviours and speciation in wild populations using social network analyses (Cunningham *et al*. 2018, McDonald *et al*. 2020). Therefore, it is not only plausible but also necessary to utilise social network analyses to quantify the social contacts within populations; (ii) reliable population demography estimations are required, as population demographic factors (e.g. ASR, age structure) play important roles in shaping social contact patterns; (iii) samples of pathogens that can transmit via social contact need to be collected (STIs and non-STIs).
3. Gut microbiota is also an important avenue for future studies. There are two potential aspects: (i) as our understanding of gut microbiota in wild birds (especially shorebirds) is very limited, future work needs to expand the research scale by including more species and populations under natural conditions across a wide geographical range, on the one hand, to characterise the gut microbiota in more natural populations; on the other hand, to better assess how gut microbiota is related or influenced by phylogeny, environmental exposures, life history traits and social structures. (ii) Little is known about the natural dynamics of bacterial pathogens in wild bird gut microbiota; therefore, it is of significance to understand

the pathogenic functional profiles of gut microbial community, which will also promote our understanding of the link between social structure and disease prevalence.

Comparative study is essential to understand a key issue in evolutionary ecology and wildlife biology, yet it is challenging. However, the collaborations among organisations and field sites will prove the feasibility of these research plans. During my PhD research, I have established efficient collaboration with researchers from different organisations, where I was able to use data from populations over a wide geographical range monitored by those collaborating researchers (Chapter 3). In addition, our research group has established long-term monitoring of more than 40 populations across all continents except Antarctica. In the future, international collaborations will be a valuable approach for the advancement of our knowledge of wild bird social structure monitoring and the related disease processes.

Overall, my studies have expanded our understanding of mating decisions and provided a theoretical relationship between social structure and disease-related processes in plovers, but major questions remain. Future empirical studies monitoring social behaviour in the wild population, as well as mapping the interactions between social structure and disease transmission dynamic, will have to follow so that our understanding of this exciting field progresses. This field will benefit much from experimental studies, long-term studies of wild populations and phylogenetic comparative analysis.

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Appendix I | Association of insularity and body condition to cloacal bacteria prevalence in a small shorebird

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Author's Contributions

J.F., J.S. and T.S. conceived and designed the research;
J.O.V. conducted the data analysis and wrote the paper;
J.M.P., M.C., A.P.-H. and G.T. planned the fieldwork and collected the samples;
all authors contributed substantially to revisions of the paper.

Abstract

Do islands harbour less diverse disease communities than mainland? The island biogeography theory predicts more diverse communities on mainland than on islands due to more niches, more diverse habitats and availability of greater range of hosts. We compared bacteria prevalences of *Campylobacter*, *Chlamydia* and *Salmonella* in cloacal samples of a small shorebird, the Kentish plover (*Charadrius alexandrinus*) between two island populations of Macaronesia and two mainland locations in the Iberian Peninsula. Bacteria were found in all populations but, contrary to the expectations, prevalences did not differ between islands and mainland. Females had higher prevalences than males for *Salmonella* and when three bacteria genera were pooled together. Bacteria infection was unrelated to bird's body condition but females from mainland were heavier than males and birds from mainland were heavier than those from islands. Abiotic variables consistent throughout breeding sites, like high salinity that is known to inhibit bacteria growth, could explain the lack of differences in the bacteria prevalence between areas. We argue about the possible drivers and implications of sex differences in bacteria prevalence in Kentish plovers.

Keywords

avian pathogens, enterobacteria, pathogen transmission, Salmonella, sexually transmitted diseases (STD), waders

Introduction

Understanding how biological diversity stablishes and evolve has been a tradition in modern ecology [1, 2]. Insights of historic and contemporary research have led ecologists to develop a number of biodiversity theories that are intended to help us predict biodiversity in a given space and/or time. According to the theory of island biogeography, landscape structure shapes species' abundance, where species richness increases as a function of the area sampled [3]. Along a gradient of ecosystems of increasing size, the number of species inhabiting those ecosystems will increase rapidly at first, but then the pace slows down for the larger ecosystems [3, but see limitations, 4]. Island biogeography theory has been mainly built upon the study of macroorganisms, with very little consideration towards the biogeography of microorganisms. In fact, whether microbial biogeography should be considered as a discipline has been subject of debate, because it has long been suggested that organisms smaller than 1 mm have a cosmopolitan distribution [5]. However, different studies have documented spatial and temporal structuration of microbial diversity [6, 7]. For example, positive taxa-area relationships have been found in free-living bacteria [8], as well as a reduction in bacterial diversity across islands of decreasing sizes [9].

Symbiotic organisms are in a close and necessary association with other organisms, through either mutualistic, commensal or parasitic associations [10]. This co-dependant interaction adds an important layer of complexity to the island biogeography theory, mainly for including variables from the host that could also be affected by insularity. For example, animals from islands have been proposed to have weaker immune defence, attributed to the founder effect during colonisation and to island environments being relatively parasite poor (compared to the mainland) [3, 11, 12]. Examples of the latter include reduced prevalence and diversity of blood parasites and fewer feather lice species in Macaronesian blackcaps (*Sylvia atricapilla*) [13, 14] and reduced viral pathogen diversity and abundance in insular black-spotted pond frogs (*Pelophylax nigromaculatus*) compared to the mainland [15]. However, contrasting results could be found for other host species and microorganisms studied [16].

Studies investigating variation in microorganism prevalence, not microorganism diversity, in relation to area sampled have been considerably less common. Microorganism prevalence, here defined as the percentage of individuals of a population infected with a given microorganism, will depend importantly upon two variables: (i) the ability of the host to defend against the infection, and (ii) on the ability of the microorganism to infect the host. As mentioned before, insularity is thought to, at certain extent, shape immune function because after many generations exposed to low pathogen pressure and diversity, selection favours a reduction in the energy invested in maintaining a robust immune function [17]. However, changes in immune parameters in response to insularity are not as straightforward as initially thought, as Matson *et al*. [18] found that in bluebirds (*Sialis sialis*) the immune response was stronger in island than in the mainland. Lobato *et al*. [19] investigated two immune components in bird assemblages from two islands and mainland in Africa,

finding that acquired immunity was lower on islands but no differences were seen in the innate immunity. The high microorganism diversity expected in mainland [9] increases the chances of hosts of encountering strains of microorganism of high virulence, that could rapidly spread out across a population and elevate prevalence at a given sampling time [20]. Also, the force of infection (the rate at which susceptible individuals become infected in a population) is expected to increase with population size in the case of having many susceptible hosts present in a large population at any given time, and because in this ecosystem the number of contacts between infected and susceptible individuals is likely to also increment [21–24]. Conversely, in simplified ecosystem (i.e. islands) the reduced pathogen pressure compared to mainland would suggest, in general, lower levels of pathogen prevalence than in the mainland [25–27]. However, to our knowledge just a handful studies have tested aspects of these predictions, and to date there is very little known on how prevalence of bacteria could be affected by insularity.

Here, we tested for the first time the influence of insularity on the transmission patterns of the bacteria *Campylobacter*, *Salmonella* and *Chlamydia* in four populations of Kentish plover (*Charadrius alexandrinus*). These bacteria are known for its importance in wildlife and public health, being usually commensal in poultry but a common cause of gastrointestinal and respiratory disease in wild birds [28, 29]. Kentish plover is a small shorebird, ideally suited for this purpose because breeds all across Eurasia and Macaronesia (Fig 1), islands where they are year-round residents and represent populations genetically distinctive from the mainland [30]. We compared the cloacal bacteria prevalence of two island populations from Cape Verde and Canary Islands, and two populations breeding in continental Spain (Table 1). Because these bacteria could provoke disease, we also investigated the effect of insularity and bacteria infection on body condition. Based on previous evidence, we predicted (i) a higher prevalence of infection in mainland than in insular populations; (ii); bacteria infection will negatively affect the host's body condition [31, 32]; and as possible consequence of the previous two, (iii) birds from islands will have better body condition than those from mainland [33]. Last, because cloacal transmission of bacteria seems to negatively affect females [35], we predicted that (iv) females could present higher prevalences than males.

Fig 1. Sampling locations of the four Kentish plover populations. Two on mainland and two on islands: (A) Rice fields of Doñana, and (B) Salina la Esperanza, Cádiz, both in Spain; (C) Lanzarote, Canary Islands, and (D) Maio, Cape Verde.

Table 1. Number of birds examined and infected in mainland and island populations of Kentish plover.

	Population	*n*	*Campylobacter*	*Chlamydia*	*Salmonella*	Pooled infection
Island	Maio, Cape Verde	88	2	5	10	17
	Lanzarote, Canary Islands	27	0	0	3	3
	Total	115	2	5	13	20
Mainland	Doñana, Spain	52	1	1	10	12
	Salina la Esperanza, Cádiz, Spain	52	1	4	10	15
	Total	104	2	5	20	27

Materials and Methods

Ethics

All necessary permits were obtained for the described field studies. Salina la Esperanza: permit granted by the University of Cádiz, Cádiz Bay Natural Park and Animal Health authorities in compliance with Spanish laws (number 2019-/2979/4202/Bc/EA 3619). Doñana: permit granted by the CSIC Ethics committee and Animal Health authorities in compliance with Spanish laws (number 2011_02 21/02/2012/77). Lazarote: permit granted by the Council of Land Usage, Sustainability and Security, Vice-Ministry of Environment, Canary Islands (number 2016/3646). Maio: permit granted by the General Directory of Environment, Cape Verde (number 33/2014).

Bird species and sampling locations

We captured, ringed, weighed and morphometrically measured breeding Kentish plovers from two different locations in mainland (southern Spain) and from two Macaronesian islands (Table 1, Fig 1). One mainland Kentish plover population was studied in the largest area of rice fields in Spain (36,000 ha) located in a reclaimed marshland behind Doñana National Park, Spain (37°07'08.3"N 6°06'33.7"W). Fieldwork was done in July 2015 at four sites during the peak of the breeding season. The other mainland population bred in a 35-ha saltpan in the Cádiz Bay Natural Park, Puerto Real, Spain (36°30'36.0"N 6°09'20.8"W). Fieldwork was conducted in April–June 2015. Among the island populations investigated, we studied Kentish plover on Lanzarote, Canary Islands (29°03'36.1"N 13°36'24.9"W). Lanzarote is the easternmost island of the archipelago, separated by approx. 120 km from North Africa and 1,000 km from the Iberian Peninsula. Fieldwork was conducted during the breeding season in April–June 2016, monitoring five sites around the island with different environments: saltpans, sandy beaches and semi-desert rocky areas. Lastly, we studied the Kentish plover population in Maio, Cape Verde (15°09'16.6"N 23°11'39.4"W), one of four Sotavento Islands in the archipelago, located at approx. 650 km from West Africa and 2,900 km from the Iberian Peninsula. Approximately 100–200 pairs bred in Maio around areas of saline lakes and saltpans of approx. 100 ha and surrounded by sandy shores. Three sites were monitored during September–November 2015. Kentish plovers present high breeding-site fidelity [35, 36] whereas during winter the birds from mainland Europe move to SW Europe and W African [37]. Kentish plover from Maio and Lanzarote are year-round residents but eventually could move between islands, particularly in the population of Lanzarote (TS, and GT pers. obs). All field procedures complied with the laws and approved by the ethics committees of the corresponding countries.

Bacteria sampling and laboratory diagnosis

Our analyses were focused on three bacteria genera of renown importance for wildlife and public health. *Campylobacter* and *Salmonella* are gram-negative bacteria from the Enterobacteriaceae family and often found as commensal microbiota in avian hosts [38, 39]. Commensal strains result from bacterial adaptation to specific hosts [28, 40]. In poultry, many specific strains are recognised as commensal such as *Campylobacter jejuni* ST-104 (ST-21 CC) in broiler [29], but in Kentish plover it is unknown whether there are species-specific strains and to what degree these strains are commensals or can harm host health. Nevertheless, pathogenic strains like *Campylobacter lari* or *Salmonella typhimurium* have been associated with gastrointestinal disease in poultry and in wild birds [29, 30, 41] and are also a latent epidemiological problem causing foodborne disease worldwide [42]. Although these bacteria are typically acquired through an oral-fecal route by ingesting contaminated food or water, evidence shows transmission after copulation, either through direct cloacal contact or due to ingestion of bacteria during post-copulatory preening [43, 34]. *Chlamydia*, on the other hand, are sexually transmitted bacteria and species like *Chlamydia psittaci* may cause chlamydiosis in birds and the zoonosis psittacosis (if transmitted to humans by contaminated aerosols), both being a systemic disease often linked to mortalities [44, 45].

Bacteria sampling took place at capture by gently introducing a sterile cotton swab into the bird's cloaca. Swabs were stored in phosphate-buffered saline (PBS) buffer at -20ºC in the field, and then in the laboratory at -80ºC until further analysis [46]. DNA extraction was done using the Maxwell® 16 Buccal Swab LEV DNA Purification Kit following the manufacturer's protocol. Detection of *Campylobacter* was based on amplification of a DNA segment within the *flaA* short variable region (SVR) of *Campylobacter jejuni* or *C. coli*, according to Ridley *et al.* [47]. *Chlamydia* detection centred on amplifying the IGS region and domain I of 23S rRNA gene, following Nordentoft *et al*. [48]. *Salmonella* detection used primers specific for the *invA* gene, as described by Rahn *et al*. [49]. In brief, real-time PCR assays were conducted with 5 µL of 2 × Rotor-Gene SYBR Green PCR Master Mix, 7 µL of RNase-Free water, 1 µL of primers, and 2 µL of DNA extract. Thermal conditions for PCRs were as follows: initial activation for 10 min at 95ºC, PCR cycling for 15 sec at 95ºC, for 30 sec at 59ºC and for 30 sec at 72ºC (for *Chlamydia*) or 30 sec at 95ºC, for 15 sec at 54ºC and 20 sec at 72ºC (for *Salmonella*) for 45 times, and melting curve were obtained by lowering the temperature from 90ºC to 75ºC, descending by 0.3ºC each step. We used DNA from *Chlamydia psittaci* and *Salmonella typhimurium* as positive controls in each reaction plate. For *Campylobacter* cycling was for 10 sec at 95ºC, for 6 sec at 50ºC and for 6 sec at 72ºC for 35 times, and melting curve were obtained by lowering the temperature from 90ºC to 50ºC, descending by 2.2ºC each step. The positive controls used were *Campylobacter jejuni* and *C. coli*. Negative controls were included in each plate.

Statistical analyses

Our predictions were tested by running Markov chain Monte Carlo simulations for generalized linear mixed models using the R package 'MCMCglmm' [50]. Differences in insularity were tested by running four models: one for each bacteria type and one for the prevalence of the three bacteria combined (pooled bacteria infection). The models had bacteria infection (binomial variable: infected/un-infected) as response variable, and insularity (binomial variable: island/mainland) and sex (binomial variable: female/male) as fixed factors as well as the two-way interaction between these two variables. Date of sampling (Julian date) and sampling site were added as random terms. Sampling site corresponded to the sites sampled within each location: four in Doñana, one in Salina La Esperanza, five in Lanzarote, and three in Maio. We used parameter expanded priors for the random effects (list(V = diag(1)*0.02, nu =7)), and fixed effect priors for binary responses i.e. fixing the residual variance at 1 (list(V = diag(1), nu = 0.002, n=1, fix=1)). Models were run across 1,000,000 iterations with thin of 600 and a burn-in of 1,500. These values were determined based on model convergence and autocorrelation levels assessed through the Gelman-Rubin test [51], and trace graphs and the 'autocorr' function, both implemented in the R package 'coda' [52]. In all four models, the potential scale reduction factor was 1.01 or lower, which is below the threshold of 1.1 indicating good model convergence. Autocorrelation was also low, always below the threshold of 0.1 [50].

Body condition was estimated using the scaled mass index proposed by Peig and Green [53], consisting on standardizing body mass for a given size using a body linear measurement (here, wing length). This analysis was conducted only for the pooled bacteria prevalence due to the very low prevalences of *Campylobacter* and *Chlamydia* (see results). This model was run with a Gaussian error distribution and had body condition as response variable and infection status, insularity and sex as fixed factors and their two-way interactions. Date of sampling and sampling site were added as random terms. We used parameter expanded priors for the random effects (same as above) but inverse gamma priors (list(V = 1, nu = 0.002)) for the residuals and normal distributions centred on zero with large variances as fixed effects priors (default prior in MCMCglmm). This model was run across 1,000,000 iterations with thin of 600 and a burn in of 1,500. Here, the potential scale reduction factor was 1.001 or lower and the autocorrelation was not higher than 0.03 [50]. In this analysis 11 Kentish plovers were excluded from the model because wing length, body mass or both measurements were not available. MCMCglmm results are expressed as posterior mean, lower and upper 95% credibility intervals, and significance as a pMCMC value. All statistical analyses were conducted in R v3.3.3 [54].

Results

Forty-seven out of 219 birds sampled were infected (21.5%). The highest prevalence was recorded in *Salmonella* (15.1%) followed by *Chlamydia* (4.6%) and *Campylobacter* (1.8%), with no birds

presenting mixed infections. Bacteria infection was spread out in most populations (Table 1) except on Canary Islands where only *Salmonella* was found (Table 1).

Bacteria prevalence was always higher in birds from mainland than from islands, however this difference was non-significant. Although a trend appeared when infection was pooled together, showing nearly significant higher prevalence of infection in mainland ($P = 0.077$; Fig 2, Table 2). Females had a higher prevalence of *Salmonella* than males, and the same pattern was found when all bacteria were pooled together (Fig 2, Table 2). The interaction between insularity and sex was not significant (all cases $P > 0.05$), so insularity did not affect bacteria prevalences between the sexes.

Fig 2. Differences in bacteria prevalence between populations. Prevalence of cloacal bacteria infection between a) mainland and island and b) male and female Kentish plovers. *Indicates a statistically significant difference of $P < 0.05$

Table 2. Infection of (a) *Campylobacter*, (b) *Chlamydia*, (c) *Salmonella* and (d) all bacteria combined in relation to insularity and sex in Kentish plovers (*n* = 219). Residual variances were fixed at 1. Significant effects in bold.

		95% credibility intervals		
	Post. mean	Lower	Upper	*P*
a) Intercept	-5.196	-7.807	-2.984	< **0.001**
Insularity (island)[a]	0.002	-3.833	3.726	0.995
Sex (males)[b]	0.533	-2.906	4.687	0.754
Insularity (island)[a]*sex (males)[b]	-0.324	-5.604	4.788	0.876
Random				
Site	0.027	0.006	0.063	
Date	0.028	0.007	0.066	
b) Intercept	-3.257	-4.519	-2.139	< **0.001**
Insularity (island)[a]	-0.945	-3.140	1.140	0.360
Sex (males)[b]	-1.434	-4.541	1.275	0.318
Insularity (island)[a]*sex (males)[b]	2.276	-1.385	6.014	0.197
Random				
Site	0.029	0.006	0.071	
Date	0.028	0.006	0.064	
c) Intercept	-1.349	-1.991	-0.630	< **0.001**
Insularity (island)[a]	-0.842	-1.958	0.219	0.129
Sex (males)[b]	-1.331	-2.584	0.094	**0.043**

Insularity (island)[a]*sex (males)[b]	0.520	-1.272	2.644	0.578
Random				
Site	0.027	0.006	0.061	
Date	0.030	0.006	0.076	
d) Intercept	-0.887	-1.53	-0.230	**0.008**
Insularity (island)[a]	-0.920	-1.939	0.110	0.077
Sex (males)[b]	-1.248	-2.526	-0.146	**0.030**
Insularity (island)[a]*sex (males)[b]	0.980	-0.693	2.678	0.252
Random				
Site	0.032	0.006	0.078	
Date	0.030	0.007	0.074	

[a]Relative to mainland

[b]Relative to females

Body condition was not significantly affected by the presence of the three bacteria (Fig 3, Table 3). Females were heavier than males in the mainland while on islands no sex differences in body condition were found (Table 3).

Fig 3. Variation in body condition in Kentish plovers. Scaled mass index in male and female Kentish plovers from mainland and islands (females and males from mainland weighed on average [mean ± standard deviation] 42.6 ± 2.9 and 40.5 ± 2.1 g, respectively, while on islands, females and males weighed 41.0 ± 2.8 and 41.0 ± 3.1 g, respectively). Medians, upper and lower quartiles are shown. Whiskers indicate minimum and maximum values and circles outliers. **Indicates a statistically significant difference of $P < 0.01$

Table 3. Factors affecting the body condition in Kentish plovers (n = 208). Eleven birds were excluded from the model. Significant effects in bold.

		95% credibility intervals		
	Post. mean	Lower	Upper	*P*
Intercept	42.656	41.802	43.486	< **0.001**
Pooled bacteria infection	-0.195	-1.578	1.151	0.789
Insularity (island)[a]	-1.815	-2.964	-0.639	**0.001**
Sex (males)[b]	-2.196	-3.445	-1.029	**0.001**
Pooled bacteria infection*Insularity (island)[a]	0.958	-0.888	3.081	0.342
Pooled bacteria infection*sex (males)[b]	0.198	-1.730	2.407	0.841

Insularity (island)[a]*sex (males)[b]	2.236	0.702	3.838	**0.006**
Random				
Site	0.028	0.006	0.071	
Date	0.029	0.006	0.067	
Residual	8.047	6.529	9.587	

[a]Relative to mainland

[b]Relative to females

Discussion

Our results showed that *Campylobacter*, *Chlamydia* and *Salmonella* were widespread among most Kentish plover populations and similarly prevalent in mainland and islands. Female Kentish plovers had a higher *Salmonella* prevalence than males, a pattern also found when the infection of the three bacteria was combined together. Lastly, we showed that body condition was not related to infection of the three bacteria but to sex and insularity, with a higher body condition found in females and in birds from the continent.

Insularity

We found similar *Campylobacter*, *Chlamydia* and *Salmonella* prevalences in insular and mainland bird populations. One reason of our findings in *Salmonella* could originate from the fact that infection with this genus of bacteria, as most shorebird microbiota, depends on the environmental availability of the bacteria [55]. Although Kentish plovers in our study bred in completely different landscapes (i.e. islands vs. mainland), the breeding sites were relatively similar in that involved lands of high salinity, scarce vegetation and close to saline water bodies. High levels of salinity could consistently constrain bacteria acquisition throughout sampling locations because salinity is a well-known inhibitor of *Salmonella* and *Campylobacter* growth [56, 57]. Perhaps the exception to this was the population from Doñana that bred near brackish water. Interestingly, the percentage of infected birds in Doñana was equal to those in Cádiz (19.2%) but higher than in Lanzarote and Maio (respectively, 11.1% and 11.4%). The animal diversity in mainland increases the probability of encountering animals hosting *Salmonella* infection that could later be acquired by Kentish plovers [58], and thus is a possible reason of the close to significant higher prevalence of *Salmonella* in the continent. However, a possible counter argument is that hot environments (25-35 degrees C) with high relative humidity such as the islands of Maio and Lanzarote, provide suitable conditions for a longer persistence in the environment of the bacteria [59], increasing the potential of between-individuals bacteria transmission through ingestion of bacteria from feathers during preening, posterior to, for example, belly-soaking [more frequent in hot environments, 60] or direct contact of individuals (e.g. copulation) [34]. *Campylobacter* may be also acquired from the environment and thus could be affected by the same factors described for *Salmonella* [61]. However,

Campylobacter is much more susceptible to environmental conditions, requiring, for example, microaerophilic conditions to proliferate [57, 62]. Another reason of such low prevalences found (1.8%, 4 infected out of 219 birds) could be because direct PCR detection from feaces can be problematic compared to enrichment and culture, regarded as the gold standard for *Campylobacter* detection [63]. Although *Campylobacter* prevalences in the wild are medium to high and around 75% in shorebirds [64], a previous study investigating the prevalence of *Campylobacter* spp. in Kentish plovers failed to find any infected individual out of 12 tested [65]. In addition, low prevalences, as found in *Campylobacter* and *Chlamydia*, may make more difficult to detect differences in prevalence between populations. This is particularly important for bacteria like *Chlamydia*, that is mainly horizontally transmitted by direct contact between infected individuals [66].

Sex-specific bacteria prevalence

Bacteria prevalence was significantly female-biased when *Salmonella* infection and all the bacteria species were analysed together. These sex differences were independent of insularity. In addition to potential differences in the ecology of the different bacteria genera, the very low prevalences of *Chlamydia* and *Campylobacter* may explain why our results only approached significance for *Salmonella*. Studies of sex-specific parasite infection (as general term) have shown great heterogeneity in their patterns and are rather scarce in terms of bacteria presence. One study investigating pathogen prevalence in the island populations of Berthelot's pipit (*Anthus berthelotii*) found no sex differences of infection with pox virus, *Plasmodium* and *Leucocytozoon* [67]. Another study found that females had higher prevalence of cloacal bacteria than males in alpine accentor (*Prunella collaris*) [68], while bacteria richness did not vary with sex in blue tits (*Cyanistes caeruleus*) [69]. This heterogeneity may be due to many non-exclusive factors, including differences in immunocompetence and behavior between host sexes as well as differences in the ways of transmission between the pathogens studied [70–72]. The immune system plays an important role in pathogen defense hence if sex-specific differences in immunocompetence exist, we could expect unbalanced infection. However, although a recent meta-analysis showed in general no sex differences in immune capacity across animals [including birds, 73], the literature available shows plenty of variation (i.e. female and male biases) at species and population level that has not yet been explained [74–77].

Body condition

Recent advances in methods of microorganism detection have shown that wild animals often are natural reservoir of pathogenic microorganisms without any apparent health cost [38, 78, 79]. The relationship between bacteria infection and body condition could be difficult to untangle because

one could argue that individuals in poor body condition would be more prone to infection, however, this is more likely to happen when access to food is reduced and immunity also gets compromised [80, 81]. Nevertheless, the consistent presence of these bacteria that we found throughout the locations and populations of Kentish plover, in addition to the lack of impact on body condition, suggests that these shorebirds are natural reservoir of *Campylobacter*, *Chlamydia* and *Salmonella*. However, these bacteria have great diversity of strains with different pathogenicity and the impact of parasites on host health, survival and life history is difficult to demonstrate based on observational studies and always require experimental manipulation of parasite prevalence or intensity of infection [82, 83]. Further studies are needed to determine whether positive birds harboured strains distinctive and specific to Kentish plover, or strains of other species that have recently adapted to this host [28]. Contrary to our expectations, birds from mainland had better body condition than those from islands. Animals living on islands are exposed to low interspecific competition for food [84]. In addition, the tropics lack of well-defined seasons, with rather stable temperatures during the day and night, and predictable foraging conditions. Such conditions could prevent birds from fuelling up excessively and store energy as fat because of the constant food availability. Also, animal in tropics tend to have slower basal metabolic rates, which imply lower caloric requirements [85]. On the other hand, birds from mainland are exposed to more variable environmental condition like lower temperatures at night, that might translate into higher food consumption during the day [86]. Interestingly, only birds from mainland had sex differences in body condition. Sex differences in body mass or body condition during breeding usually occur previous to the egg-laying stage, where females increase their body mass, and then later during the nesting and brood care stage, where the sex that provides most of the care will see the detrimental effects on body weight [e.g. 87–89]. If we take this into consideration, in addition to previous studies in different continental Kentish plover populations describing a polyandrous mating system [90], we could argue that a polyandrous mating system could explain why only in mainland we found that females were heavier than males, because in polyandry males provide the brood case. However, this postulate cannot be confirmed because to date, no empiric studies have investigated the mating system of the mainland populations here studied.

Conclusion

Although in a relatively low prevalence, our study shows that *Campylobacter*, *Chlamydia* and *Salmonella* were widely present across Kentish plover populations, placing it as possible natural reservoirs of these bacteria. Contrary to our expectations, the three bacteria examined were equally prevalent on mainland and on island populations. Insularity and the sex of the host were important variables determining the bird's body condition, but these patterns were difficult to interpret. Positive relationships between geographical size and animal, plant and bacteria diversity have been

reported [e.g. 9, 91]. In the case reported here, it is possible that bacteria infection in hosts do not directly depend on geographical size because of the added level of complexity of including the many variables of the host that could also be affected by insularity. We emphasize on expanding research on bacteria infection in wild birds from an ecological point of view, necessary to further understand the potential impact of social interactions and mating system structure on sexual differences in the prevalence of cloacal bacteria.

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Appendix II | The allocation between egg size and clutch size depends on local nest survival rate in a mean of bet-hedging

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Author's Contributions

ZS, LX, YL conceived the study, conducted the analyses, and wrote the manuscript;
PQ, NH, QH contributed to data collection and result interpretation;
TS participate in project design, advised on data analyses and manuscript writing and reviewed drafts of the paper;
YL and ZZ coordinated the study system of the plovers, conceived the research ideas;
All authors reviewed the manuscript and gave final approval for publication.

Abstract

The allocation of resources between offspring size and number is a central question of life-history theory. Although several studies have tested the existence of this trade-off, few studies have investigated how environmental variation influences the allocation of resources to offspring size and offspring number. Additionally, the relationship between population dynamics and the offspring size and number allocation is far less understood. Here, we investigate whether resource allocation between egg size and clutch size is influenced by the ambient temperature and whether it may be related to apparent nest survival rate. We measured 1548 eggs from 541 nests of two closely related shorebird species, the Kentish plover (C*haradrius alexandrinus*) and the white-faced plover (*C. dealbatus*) in China, in four populations that exhibit contrasting ambient environments, and we weighed females, monitored nest survival, and calculated the variance of ambient temperature. Although we found that egg size and clutch size were all different between the four breeding populations, the reproductive investment (i.e., total clutch volume) was similar between populations. We also found that populations with a high survival rate had relatively larger eggs and a smaller clutch than populations with a low nest survival rate. The latter result is in line with a conservative/diversified bet-hedging strategy. Our findings suggest that plovers may increasing fitness by investing fewer, larger or many, small according local nest survival rate to make a similar investment in reproduction, and thereby may have an impact on population demography.

Keywords

resource allocation, bet-hedging strategy, nest survival rate, ambient temperature, shorebirds.

Introduction

Understanding the relationship between the size and number of progeny is a central question of life-history theory (Clutton-Brock 1991, Stearns 1992). Indeed, egg and yolk mass can strongly affect the quality of offspring across a variety of taxa (Krist 2011). Larger eggs have a higher hatching rate (Saino, *et al*. 2004) and higher nestling survival rate than smaller eggs (Arnold, *et al*. 2006, Williams 1994), and nestlings from heavier eggs are also more likely to have higher fitness after fledging (Krist 2011, Smith, *et al*. 1998). However, given finite resources for breeding females, life-history theory predicts that larger eggs or larger offspring are expected to be produced at the expense of fewer progeny (Clutton-Brock 1991, Stearns 1992). Several interspecies (Blackburn 1991, Charnov, *et al*. 2006, Christians 2000) and intraspecies (Fleming, *et al*. 1990, Williams 2001) studies have shown negative relationships between clutch size and egg size, which means females will allocate resources between the number and quality of offspring. However, the existence of the number-size trade-off should not only be tested (Hein, *et al*. 2018, Lessells, *et al*. 1989), but should also be explored under the impacts of different environment and/or maternal conditions, and their consequences on resource allocation strategy.

In any given reproduction attempt, life-history theory suggests that parents should make a fundamental decision about how to allocate resources between egg size and clutch size (Stearns 1992). A handful of studies show that this size-number trade-off strategy may vary with environmental conditions and may interact by maternal effects (Crean and Marshall 2009). Individual breeding in the lower latitudes produces fewer larger offspring than high latitudes (Fleming and Gross 1990) or drought year (Abell 1999). However, there has been no attempt to explain the relationship between the offspring size-number trade-off and population dynamics, such as nest survival rate.

The nest survival rate in many species highly depends on the predation risk and anthropogenic disturbance of both adults and the nest. Predator effects on prey demography have traditionally been ascribed to direct killing (Preisser, *et al*. 2005, Que, *et al*. 2015), but predators and human may also have indirect effects on the breeding decisions of prey species (Creel, *et al*. 2007, Travers, *et al*. 2010). Clutch size may decrease under a low nest survival rate as a low investment in reproduction could provide parents with the opportunity of surviving until the next reproductive event or re-laying a new nest (Doligez, *et al*. 2003). Because of the optimal investment theory which suggests that females with more resources should produce offspring of a uniform size but in higher numbers (Hendry, *et al*. 2001, Smith, *et al*. 1974), variation in clutch size is considered as a primary adaptation strategy to local nest survival rate (Slagsvold 1982). However, small clutch size cannot explain the adaptation of individuals who live under high nest predation risk over the long-term. Despite the strong support for the existence of a size-number trade-off in both model and empirical data in birds (Plaistow, *et al*. 2007, Williams 2001), studies on nest predation risk

and its influence on breeding performance usually ignore the variation in resource allocation trade-off between egg size and clutch size.

Recent research indicates that the resource allocation between egg size and clutch size should vary according to the theory of bet-hedging (Olofsson, *et al*. 2009). The idea behind a bet-hedging strategy is that an individual has to lower its variance in fitness between years to maximize its long-term fitness (Einum, *et al*. 2004a, Olofsson, Ripa, *et al*. 2009). There are two fundamentally different forms of bet-hedging strategy: diversified and conservative. Diversified bet-hedging involves managing risk by increasing variation and spreading the risk. For example, to adapt to the harsh and unpredictable environment, individuals tend to produce more offspring so that potential adaptive phenotypic variation can increase the opportunity that at least some offspring can survive (Simons 2007). In contrast, a conservative bet-hedging strategy, in a stable environment, manages risk by investing more in fewer offspring (Einum and Fleming 2004a, White, *et al*. 2013). For instance, some individuals produce fewer offspring but invest more in each individual to increase the probability that each offspring will be well-equipped to handle a range of conditions. Theoretical studies indicate that individuals would develop a diversified bet-hedging breeding strategy to spread the risk under unpredictable environment (Einum and Fleming 2004a, Marshall, *et al*. 2008, Olofsson, Ripa, *et al*. 2009), but direct evidence for an adaptive origin of this behavior is lacking (Beaumont, *et al*. 2009).

Here, we test the bet-hedging theory on variation in egg size and number using four *Charadrius* populations breeding in different environments (Paisha Island: a tropical coast population, Bohai Bay: a temperate coast population, Taitema Lake: a desert inland lake population, and Qinghai Lake: a plateau endorheic alkaline lake population, Figure 1). Compared with altricial species, the relationship between chick fitness and egg size seems to be clearer in precocial species (Williams 1994). Due to the lack of intensive parental care, yolk mass may be the primary parental investment in precocial species (Einum, *et al*. 2004b, Jetz, *et al*. 2008). Hence, the resource allocation between egg size and number should be more important and closely associated with reproductive success (Balasubramaniam, *et al*. 2016). The climate in Paisha Island is typical of a tropical monsoon, and it has the highest daily temperature among the four study sites, with an average temperature of 26.98 ℃ (Figure 1). On the other three sites, Bohai Bay with a temperate climate, which has the highest unpredictability in the daily temperature of the four sites (Figure 1), Qinghai Lake is an endorheic alkaline lake at 3200 m above sea level, and has the lowest temperature among the four study (Figure 1) and Taitema Lake is located in the south-east of the Taklimakan Desert, and has the highest daily temperature difference during the breeding season (Figure1). Hence, we predicted if plovers allocate their resource between egg size and clutch size depend on ambient temperature, plovers breeding in Paisha Island have developed a conservative bet-hedging strategy which produce larger eggs of relatively small clutch size, and population

breeding in the other three sites will produce a larger clutch comprising of relatively small eggs, which presents a diversified bet-hedging strategy in order to spread the risk.

Methods

Study species and working sites

We used four populations of two related shorebird species, the Kentish plover (C*haradrius alexandrinus*) and the white-faced plover (*C. dealbatus*), to test variation in the resource allocation between egg size and clutch size in different temperatures. The latter species was originally described as a subspecies of *C. alexandrinus*. However, recent studies show that *C. dealbatus* is an incipient species of *C. alexandrinus*, which shows local adaptation to subtropical coastlines (Sadanandan, *et al*. 2019, Wang, *et al*. 2019a, Wang, *et al*. 2019b). Breeding records of *C. dealbatus* have been reported along the coast of China from Fujian Province to Hainan Island, and in south-central Vietnam (IUCN 2017), and the Kentish plover, *C. alexandrines* breeds in coastal areas and inland lakes in Europe, Asia and North Africa (Wiersma, *et al*. 2016), and it is one of the common summer breeders along the northern coastline of China and some inland lakes (Que, *et al*. 2019, Wang, Que, *et al*. 2019b). The Kentish plover complex has long been an ecological model species to understand parental care and breeding system evolution in birds (Székely 2019, Vincze, *et al*. 2017). These two precocial plovers usually produce 2 or 3 eggs, with the egg volume of a clutch being about 49.5% (unpublished data) of the female's body mass. In China, as the white-faced plover is restricted in its range to subtropical climates (Paisha Island), comparing it with three populations of the closely related Kentish plovers, in north temperate coastal (Bohai Bay), desert inland lake (Taitema Lake) and alpine inland lake habitats (Qinghai Lake), provides a good study system to understand local adaptation and breeding strategies.

We conducted fieldwork from April to June each year from 2016 to 2019 at Paisha Island (20°54′N, 110°29′E, PI), Bohai Bay (39°14′N, 118°52′E, BB), and Qinghai Lake (36°48′N, 100°45′E, QL), and we only conducted fieldwork in Taitema Lake (39°26′N 88°12′E, TL) in 2019.

Field data collection

Egg measured

At each study sites, we measured the length and breadth (± 0.01 mm) of each egg after we found a nest. We calculated egg size using the formulas by $V = 0.51 \times \text{length} \times \text{breadth}^2$ (Hoyt 1979), and we used the average egg size of each nest in analyses. Then we captured breeding female with a walk-in funnel trap placed over the nests that had been incubated for at least seven days, and we measured female body mass. We estimated the egg-laying date by floating the eggs in lukewarm water (Székely, *et al*. 2008). We checked nests at 4-5 day intervals to estimate egg survival, and near the estimated hatching date, which was approximately after the 22nd day of incubation, we checked nests daily.

In total, we measured 1548 eggs from 541 nests: 301 nests from Bohai Bay, 83 nests from Qinghai Lake, and 139 nests from Paisha Island during 2016-2019, and 18 nests from Taitema Lake in 2019.

Nest survival rate

We measured the nest survival rate in two ways: first, we calculated the number of nests that had been destroyed or had been hatched and still hatching one day before each nest was laid, then we used the percentage of nest which not been destroyed to represent the apparent nest survival rate of each nest, and second, we used nest monitoring data for each year to estimate the nest survival rate of each population per year. The nest usually lost complete of their eggs, and we classified a 'failure' nest if it lost the entire full clutch. In total we monitored the fate of 1204 nests of four populations.

Ambient temperature

We collected meteorological data, including daily mean temperature, daily highest and lowest temperature, from the National Meteorological Information Center (http://www.nmic.gov.cn/en/) of the China Meteorological Administration. As most of the effects of temperature on egg size occur during the period of rapid yolk development (RYD)(Ardia, *et al*. 2006, Saino, *et al*. 2004), and it usually took ten days after Kentish plover courtship for females to lay their first eggs (Székely, *et al*. 1999), we calculated the average value, average daily difference and variance of temperature in the ten days before each nest's laying date. We used the average difference between the highest and lowest temperatures of each day in the ten days as the daily difference in temperature. We calculated the variance of temperature by fitting a linear model between the average temperature of each day and the number of days before the laying date, and we used the standard error of the regression as an index of the temperature variance of each plover's experience (Simerly, *et al*. 2000).

Statistical analyses

To control for seasonal changes, we used the Julian day, which we calculated as the number of days between the 1st March, as the egg-laying date. We established the laying date data by monitoring hatching or making an estimation using egg flotation within 11 days of hatching, which is supposed to be accurate (Székely, *et al*. 2008). The different of egg size among the four populations were tested by fitting GLMMs, in which laying date was entered as a fixed index and the parameter years was entered as a random effect. One of the study species, the white-faced plover C. *dealbatus* was formerly treated as subspecies of Kentish plover (Hartert, *et al*. 1915, Swinhoe 1870). Some earlier studies suggested it might be a full species based on its unique morphology and ecology (Kennerley, *et al*. 2008, Rheindt, *et al*. 2011). And this hypothesis has been lately supported by genetic works (Sadanandan, *et al*. 2019, Wang, *et al*. 2019b). Even though, *C. dealbatus* shows a low level of genome-wide divergence with *C. alexandrinus*, indicating the two taxa being a pair of incipient

species (Wang, *et al*. 2019a). In order allow comparisons of life-history traits and breeding parameters, we firstly obtained genetic relatedness of the four populations of the two species by calculating genome-wide pairwise population differentiation (*F*st) using the dataset and the method in Wang, *et al*. (2019a). Then we incorporated pairwise *F*st values as a covariance matrix (Table S1) representing the amount of shared evolutionary history between four populations and performed phylogenetic generalized linear mixed models (PGLMM). We first fit a PGLMM to estimate the influence of apparent nest survival rate and ambient environment on eggs size, as the mean of eggs size within a clutch was used as dependent variable, and nest survival rate, average temperature, daily temperature difference, temperature variance, clutch size, laying date and female body mass was entered as fixed indexes, and the parameter years was entered as a random effect.

We then estimated the influence of apparent nest survival rate, average temperature, daily temperature difference, temperature variance on clutch size. As the variable of plover clutch size is count data with a range from one to four and often under-dispersed, we fitted a generalized linear mixed-effect model (GLMM) with Conway-Maxwell-Poisson distribution (Sellers, *et al*. 2010) to analyze the influence of apparent nest survival rate, laying date, average temperature, daily temperature difference, temperature variance and female body mass on clutch size. To control the interpopulation phylogenetic relatedness, we include populations as a fixed index, and the parameter years was entered as a random effect.

We test the difference of total egg size (summation of total eggs size within a clutch) between four populations by fitting a GLMM in which laying date and female body mass enter as a fixed index, and the parameter years was entered as a random effect.

Female can allocate finite resources between egg size and clutch size (Smith and Fretwell 1974). We estimate the resource allocation variance between egg size and clutch size by calculating the relative egg size for clutch size by regressing the mean egg size and clutch size taking the residuals across all nests (Martin 2008). We applied a PGLMM to analysis effects of apparent nest survival rate, average temperature, daily temperature difference, temperature variance, laying date and female body mass on this size-number allocation strategy, and the parameter years was entered as a random effect.

We fitted the PGLMM analyses using "phyr" package (Li, *et al*. 2020) and we performed GLMM analyses using the "glmmTMB" package (Magnusson, *et al*. 2017). We conducted all statistical analyses using R version 3.4.1 (R Development Core Team; http://cran.r- project.org/).

Results

Breeding performance and ambient environment between populations

Egg size and clutch size was significantly different among the four populations (egg size: GLMM, $X^2 = 60.739$, $P < 0.001$, $n = 541$; clutch size: GLMM, $X^2 = 29.019$, $P < 0.001$, $n = 541$). In Bohai Bay, females produce the smallest egg and the largest clutch size comparing with the other three

populations (Figure 2a). Additionally, female breeding in Qinghai Lake and Taitema Lake produced relative larger egg size and smaller clutch size among the four populations (Figure 2a).

The egg size was not statistically correlated with any environmental factors, such as ambient temperature, daily temperature difference and temperature variance, however, egg size increased with female body mass (Table 1). Additionally, clutch size decreased with laying date processing and increased with average temperature increasing (Table 2). Additionally, the total egg size within a clutch has no difference between four populations (GLMM: $X^2 = 1.870$, $P = 0.600$, $n = 541$, Figure 2b).

Resource allocation between egg size and clutch size relates to nest survival

Nest survival rates were different between populations: plovers breeding in Taitema Lake had the highest nest survival rate (0.702, n = 47 nests), followed by the population in Qinghai Lake (0.458 ± 0.161, n = 106 nests), Bohai Bay (0.287 ± 0.082, n = 741 nests) and Paisha Island (0.359 ± 0.058, n = 310 nests).

We found egg size increased with elevated level of local nest survival rate (Table1, Figure 3a) and, and clutch size also deceased with elevated level of nest survival rate (Table2, Figure 3b). Additionally, the egg size-clutch size allocation strategy was positively correlated with nest survival rate, meaning that plovers produce many small eggs within a clutch at a low nest survival rate and produce fewer larger eggs when local nest survival rate is high (Table 3, Figure 5).

However, neither the ambient environment parameters (i.e. average temperature, daily temperature difference and temperature variance) nor the laying date have influence on the egg size-clutch size allocation strategy (Table 3).

Discussion

In this study, we found that four plover populations adopted different patterns of resource allocation between egg size and clutch size. However, inconsistent with our prediction, the egg size and clutch size did not show tight correlation with ambient temperature. In the Paisha Island with predictable environment, the population did not show a conservative bet-hedging strategy on egg size and clutch size. We found the plover population at Bohai Bay showed a diversified bet-hedging strategy with the smallest egg size but the largest clutch size of the study populations. In contrast, the Qinghai Lake and Taitema Lake populations produced larger eggs but smaller clutches, which is consistent with a conservative bet-hedging strategy. Additionally, we found that the different allocations between egg size and clutch size seemed to be affected by apparent nest survival in that: plovers will produce fewer but large eggs under a high local nest survival rate, and produce many but small eggs under a low local nest survival rate. In addition, clutch size decreased with the advancement of laying date and decrease in ambient average temperature.

The finding that clutch size declined with progressed breeding season is consistent with several other studies that have also found a seasonal decline in clutch size (Decker, *et al*. 2012, Rowe, *et al*. 1994). It has been suggested that individuals that breed early in the season may have greater experience than late breeders (Nol, *et al*. 1987), and eggs that are laid earlier give offspring more time before winter and migration (Winkler, *et al*. 1996). Hence, plovers that breed early in the season may invest more in current reproduction. Additionally, we found a positive correlation between clutch size and ambient temperature. Possible reasons for this are that less energy is required for thermoregulation in warmer temperatures, so more energy can be allocated toward reproduction (Meijer, *et al*. 1999), and warmer temperatures increase avian food availability (Winkler, *et al*. 2013).

The allocation of resources between egg size and clutch size was different between the four populations: the Bohai Bay population produced many small eggs, and plovers breeding in Qinghai Lake and Taitema Lake tended to produce fewer larger eggs, and Paisha Island was in the middle of these two diversified and conservative bet-hedging strategies.

The bet-hedging strategies, which suggest to manage risk by spread or pull together, are expected to evolve under conditions of unpredictable environmental variance (Einum and Fleming 2004a, Simons 2011). Although there was substantial variation in the ambient temperature between the four populations, the allocation between egg size and number was not influenced by ambient temperature, daily temperature difference or temperature consistency. Bohai Bay has the highest variance of temperature and Taitema Lake has the highest daily temperature different which suggest to be environmental unpredictable, and plover breeding here should spread the risk according a diversified bet-hedging strategy. In our study, Bohai Bay population produced the largest clutch size and smallest egg size which show a diversified bet-hedging strategy. However, inconsistent with the prediction, plover breeding in Taitema Lake show a relative conservative bet-hedging strategy which produce larger eggs but smaller clutches. Additionally, the predictable tropical environment in Paisha Island is a favorable condition for reproduction, and bet-hedging strategy according to ambient environment suggests that parents breeding here are risk averse and should produce egg larger than those unstable environment (Einum and Fleming 2004a). However, Paisha Island population is between those two bet-hedging strategies, which show the second smaller egg size and second larger clutch size among these four populations.

In our study, resource allocation between egg size and clutch size involved a bet-hedging strategy that was dependent on the apparent nest survival rate. Bohai Bay has the lowest nest survival rate reported worldwide for this species (Que, *et al*. 2015), which shows a diversified bet-hedging strategy. On the other hand, populations breeding along Qinghai Lake and Taitema Lake have relatively higher nest survival rates showing a conservative bet-hedging strategy. Despite the extremely low average temperature in Qinghai Lake and highest daily temperature different in Taitema Lake, these two populations have a relatively high nest survival rate. This may be because

of limited human habitation in these two populations: Qinghai Lake is in the Qinghai-Tibetan plateau, which is the world's highest plateau, and Taitema Lake is no man's land. For Kentish plover, since anthropogenic disturbance seem to be critical for nest survival (Que, *et al*. 2015), the absence of anthropogenic disturbance may lead to a higher nest survival rate in Qinghai Lake and Taitema Lake.

Reproductive failure caused by nest predation and anthropogenic disturbance represents an important source of natural selection (Chalfoun, *et al*. 2010, Que, *et al*. 2015, Smith, *et al*. 2012), and it significantly influences reproductive strategies (Lack 1947, Mönkkönen, *et al*. 2009). The egg mortality rate may decrease the optimal clutch size for many reasons. Skutch (1949) suggested that there is a brood-size dependent predation rate, and a smaller-sized nest is more difficult for a predator to discover (Perrins 1977, Snow 1978). Additionally, a smaller clutch size is likely to be a strategy to allow rapid re-mating and re-laying if a nest was destroyed (Dillon, *et al*. 2017, Eggers, *et al*. 2005, Zanette, *et al*. 2011). Nevertheless, study reported increasing reproductive investment with increasing nest predation rate and that parents should increase their clutch size to compensate for the decreased number of successful broods because of predation (Griebeler, *et al*. 2010). Despite the inconsistency, these studies all focus on the relationship between clutch size and nest survival rate but not the allocation between egg size and clutch size. In our study, plovers increased investment in egg size at the expense of lower numbers of eggs when the nest survival rate was high, which suggests plover could use different bet-hedging strategies between egg size and clutch size to achieve similar reproductive success.

Under higher nest survival rates, the plovers produced a small clutch of larger eggs. Under a guaranteed hatching environment, this strategy might have benefits for the long-term survival of the population. Individuals hatching from larger eggs are more likely to have greater reproductive success during their lifetime (Krist 2011, Smith and Bruun 1998) than lower quality offspring from smaller eggs (Song, *et al*. 2018) and therefore represent higher reproductive fitness for their parents. Additionally, we found that there was no difference of total eggs size, which represent an equal investment of female at one breeding attempt among populations. Despite the significant variation in egg size and clutch size between the four populations, plovers may use different resource allocation strategies between each egg size and clutch size to make a similar investment in one breeding attempt.

Conclusions

We found that plovers allocate resources between egg size and clutch size following the diversified and conservative bet-hedging strategies. Plovers breeding in a temperate environment in Bohai Bay produced a larger clutch of relatively small eggs, which presents a diversified bet-hedging strategy. Additionally, the ambient temperature did not influence resource allocation between egg size and clutch size, apparent nest survival rate did. We suggest that nest survival rate, which determines the

fate of an egg, could substantial influence the potential benefit of investing in a given egg than other environmental factors that indirectly influence the egg's fitness. This might also suggest variation in ambient temperature did not influence investment of parental resource at an early stage of breeding attempts, i.e. egg-laying, yet indeed affects investment of time allocation, such parental care behavior at incubation stage under different environments (Vincze, *et al*. 2017). Overall, our findings highlight nest survival is a critical factor that have shaped life-history characters in ground-nesting birds, and further promote understanding of the drivers of geographical variations of life-history traits.

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Availability of data and materials

The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request.

Ethical Note

This study was conducted with standard protocol (Székely, Kosztolányi, *et al*. 2008) which in accordance with the ethical standards of the Sun Yat-sen university and university of Bath. Birds were ringed and handled by trained people aiming to cause as little disturbance to birds as possible.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Relationships between egg size and clutch size, female body mass, laying date, apparent nest survival rate and temperature variables in four populations of two congeneric plovers (PGLMM).

Parameter	Estimate	SE	*Z*	*P*
Egg size (n =541)				
Intercept	6.983	0.598	11.669	<0.001***
Clutch size	-0.037	0.062	-0.587	0.557
Female body mass	0.047	0.008	6.078	<0.001***
Laying date	0.003	0.003	1.294	0.196
Ambient temperature	-0.003	0.014	-0.227	0.820
Daily temperature difference	-0.022	0.020	-1.079	0.280
Temperature variance	-0.420	0.335	-1.255	0.210
Apparent nest survival rate	0.371	0.176	2.107	0.035*

The parameter Years was entered as a random factor.

Significant relationships are indicated as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

Table 2 Relationships between clutch sizes and female body mass, laying date, apparent nest survival rate and temperature variables in four populations of two congeneric plovers (GLMM): white-faced plover at Paisha Island (PI), and Kentish plover at Bohai Bay (BH), Qinghai Lake (QH), and Taitema Lake (TL).

Parameter	Estimate	SE	X^2	*P*
Clutch size (n =541)				
Intercept	1.197	0.114		
Populations (BH as baseline)			29.019	<0.001***
QH	0.101	2.888		
TL	-0.008	0.053		
PI	-0.175	0.038		
Female body mass	-0.000	0.002	0.002	0.965
Laying date	-0.003	0.001	24.375	<0.001***
Ambient temperature	0.011	0.004	8.544	0.003**
Daily temperature difference	-0.004	0.006	0.431	0.512
Temperature variance	0.060	0.077	0.612	0.434
Apparent nest survival rate	-0.077	0.038	4.078	0.043*

The parameter Years was entered as a random factor.

Significant relationships are indicated as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

Table 3 Relationships between the egg size-clutch size allocation strategy (higher value means larger egg size with small clutch size) and laying date, apparent nest survival rate and temperature variables in four populations of two congeneric plovers (PGLMM).

Parameter	Estimate	SE	*Z*	*P*
Egg size-clutch size allocation strategy (n = 541)				
Intercept	-2.177	0.562	-3.876	<0.001***
Female body mass	0.047	0.008	6.037	<0.001***
Laying date	0.003	0.003	1.063	0.288
Ambient temperature	-0.002	0.014	-0.117	0.907
Daily temperature difference	-0.022	0.020	-1.075	0.282
Temperature variance	0.403	0.335	-1.201	0.230
Apparent nest survival rate	0.348	0.176	1.983	0.047*

The parameter Years was entered as a random factor.

Significant relationships are indicated as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

Figures

Figure legends

Figure 1: Ambient environment in four populations (GLMM, average temperature (AT): X^2 = 8309.706, $p < 0.001$; daily temperature difference (DD): $X^2 = 2291.756$, $p < 0.001$; temperature variance (TV): $X^2 = 368.019$, $p < 0.001$) in Bohai Bay, Qinghai Lake, Taitema Lake and Paisha Island. Left Y axis shows the Celsius of average temperature and daily temperature difference, right Y axis shows the variance of daily temperature within 10 days (higher means variance).

Figure 2: Differences in egg size and clutch size (a) and total eggs size (b) between populations. BH: Bohai Bay (circle), QH: Qinghai Lake (square), TL; Taitema Lake (triangle) and PI: Paisha island (inverted triangle).

Figure 3: The egg size (a) and clutch size (b) correlated with apparent nest survival rate in four populations.

Figure 4: The egg size-clutch size allocation strategy increased with apparent nest survival rate in Bohai Bay (circle), Qinghai Lake (square), Taitema Lake (triangle) and Paisha Island (inverted triangle).

Figure 1

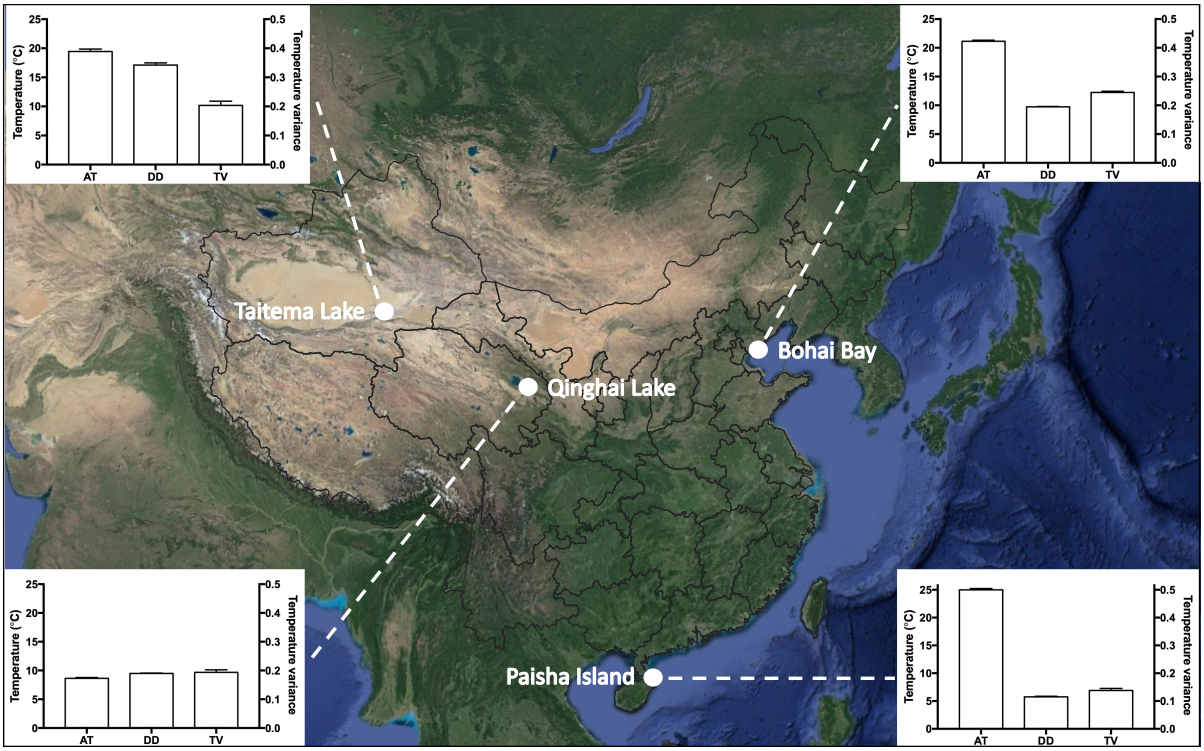


Figure 2

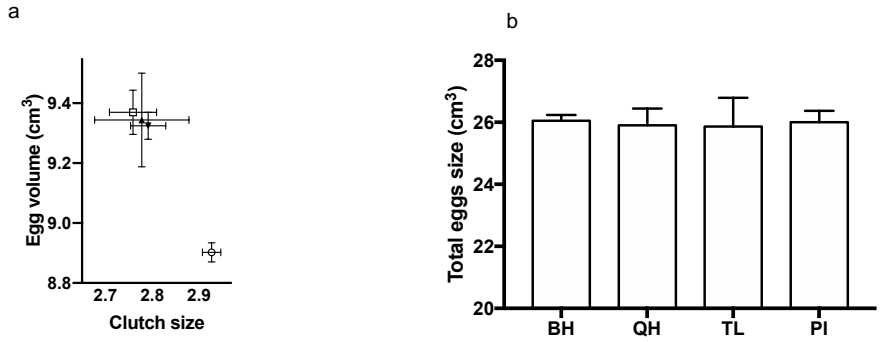


Figure 3

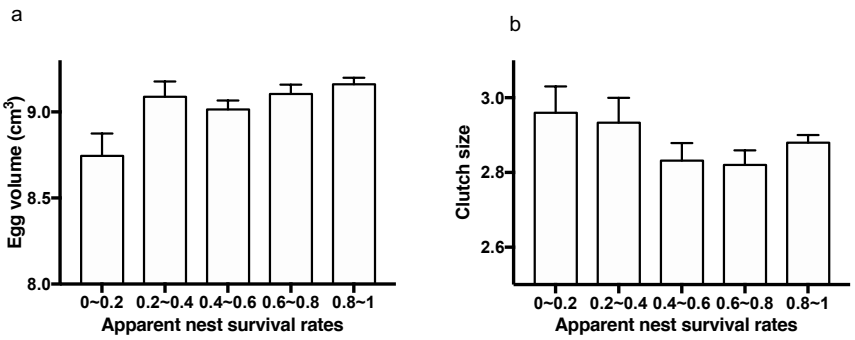
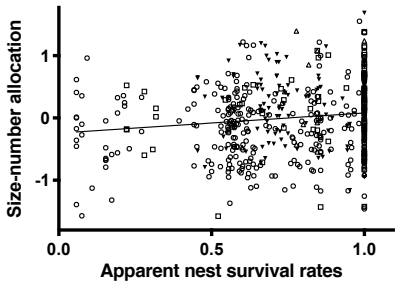


Figure 4


Size-number allocation
1
0
-1
0.0
0.5
1.0
Apparent nest survival rates

Appendix III | Timing of breeding in relation to weather in the world's plovers

Udita Bansal, Naerhulan Halimubieke, Zsolt Végvári, Afonso D. Rocha, András Kosztolányi, Cheri-Gratto Trevor, Daniel Burgas, Daniel H. Catlin, Daniel Galindo-Espinosa, Dominic V. Cimiotti, Eduardo Palacios, Jesús Domínguez Conde, Jonathan B. Cohen, Jordi Figuerola, Juan A. Amat, Maï Yasué, María de los Ángeles Hernández, María Vidal, Matthew Johnson, Mauro Mencarelli, Michael A. Weston, Michelle L. Stantial, Oscar García-Miranda, Penn Lloyd, Pinjia Que, Sama Zefania, Richard Lanctot, Sarah T. Saalfeld, Tomás Montalvo, Tamás Székely

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Author's Contributions

UB conceived the idea, assembled and cleaned the data, downloaded weather data, did the analysis, prepared the figures and tables, wrote and finalised the manuscript.

ZV downloaded weather data, reviewed drafts of the manuscript, provided supervision for analysis and interpretation of results.

AR, AK, CGT, DHC, DGE, DB, DVC, EP, JBC, JD, JF, JAA, MY, MAH, MV, MJ, MM, MAW, MLS, NH, OGM, PQ, PL, SZ, RL, STS, TM, UB, TS collected and provided the data for different populations.

NH reviewed drafts and edited drafts of the manuscript.

TS reviewed drafts of the manuscript approved the final draft and provided supervision throughout the study.

Abstract

Environmental variables, such as temperature and precipitation, are known to influence avian breeding phenology and have major implications for conservation on a warming planet. Therefore, it is important to understand the associations between local climatic conditions and breeding progression (changes in the number of nests/clutches laid across the breeding season) of birds. Studies typically investigate one population in a given study site, and thus, the general patterns are rarely uncovered. Plovers are globally distributed, ground-nesting shorebirds that live on all continents except Antarctica, breed in a wide range of environmental conditions and exhibit diverse breeding systems. Here, we capitalize on this unusual diversity, and by using data from 26 populations of 12 plover species, we investigate how ambient temperature and precipitation influence breeding progression and nest failure. We show that clutch initiation each week was best predicted by ambient temperature and precipitation in the two weeks prior to egg laying. The relationship was quadratic for temperature since the highest proportion of clutches were laid at intermediate temperatures. However, the relationship was linear for precipitation. Proportion of failed nests also exhibited a quadratic relationship with ambient temperature from three days prior to fate of the nest where it was lowest at temperatures higher than the mean. It showed a linear relation with three-day lag in average precipitation where nest failure increases with increasing precipitation. Our work indicates that changes in the environmental conditions within a breeding season can cause a drastic decrease in the number of successfully breeding plovers in a given season, due to effects on two key stages: clutch initiation and nest fate. A better understanding of the associations between local weather conditions and reproductive timing and success is useful to management of plover populations under predicted future climate scenarios.

Keywords

temperature, precipitation, plovers, global, breeding progression, clutch initiation, nest fate

Introduction

Temperature and precipitation have long been known to influence phenology, species distributions, physiology, growth, development, metabolism, and population dynamics across taxa, and have key fitness implication in animals (Pörtner, 2001; Brown *et al*., 2004; Yom-Tov & Geffen, 2006; Hansen, 2009; Zuo *et al*., 2011; van Gils *et al*., 2016). Although there is extensive research regarding the effects of weather and climatic change on biodiversity and ecological processes (Janzen, 1994; Walther *et al*., 2002; Parmesan and Yohe, 2003; Zimmer, 2004; Perry *et al*. 2005; Sunday *et al*., 2012), our knowledge remains limited in terms of the effects of changes in weather conditions over short periods of time on the reproductive traits in wild populations.

The effects of environmental conditions on breeding phenology and success in wild avian populations have received much attention (Moss, 1986; Beintema and Visser, 1998; Crick and Sparks, 1999; Parmesan and Yohe, 2003). Weather conditions often influence breeding phenology in birds, as environmental cues such as photoperiod, light intensity, temperature and precipitation affect food abundance and availability (Keast and Marshall, 1954; Immelman, 1969; Sinclair, 1978; Pienkowski, 1983; Halse and Jaensch, 1989; Perrins, 1991; Hahn *et al*., 1997; Hau, 2001; Stenseth and Mysterud, 2002; Santana *et al*., 2015). Breeding pairs synchronise the hatching of chicks with food abundance to improve offspring survival (Perrins, 1991; Székely *et al*., 1993; McKinnon *et al*., 2012). For example, temperature predicts the breeding phenology of several middle and high latitude birds such as arctic-breeding shorebirds (Kwon *et al*., 2018), Great tits (*Parus major*) (Visser *et al*., 1998) and flycatchers (*Ficedula* spp.) (Both *et al*., 2004). Similarly, rain also has been found to predict breeding initiation in birds of arid and semi-arid regions where precipitation is positively correlated with food abundance (Keast and Marshall, 1954; Immelman, 1969; Brown and Li, 1996).

Temperature and precipitation levels across the globe have changed over the past few decades due to natural and human induced causes which have in turn resulted in mistimed breeding cues. This has led to advances or delays in avian laying dates which may decouple correlations between the hatching of chicks and resource abundance, reducing productivity and offspring survival (Visser *et al*., 1998; Both *et al*., 2009; Mckinnon *et al*., 2012; Saalfeld *et al*., 2019). Aside from phenological shifts, some studies have analysed changes in other reproductive traits such as clutch size, egg volume, incubation period and nest survival in response to climatic changes (Both & Visser, 2005; Hargitai *et al*., 2016; Kwon *et al*., 2018). However, we still lack knowledge about the effects of environmental variables on the clutch initiation by birds after the gonads are prepared for mating and egg-laying. Moreover, climate change not only consists of global rise in temperatures or changes in snow melt across years, it also entails random events over shorter periods of time. These events include increases in acute precipitation events, extended periods of low precipitation, decreases in extreme low temperatures and increases in extreme high temperatures (Easterling *et al*., 2000; Meehl *et al*., 2000). These events are not necessarily captured

in the global changes in average spring temperatures, and hence their relationships with shifts in laying dates or phenological mismatches remain to be understood. It seems intuitive to expect an effect of such events on clutch initiation and also failure of nests, even if they occur after the start of the breeding season for birds since organisms are generally well-adapted to the environmental conditions they live in. Thus, it is imperative to bridge our knowledge gap regarding the effects of local environmental conditions, if any, on the initiation of clutches by birds after they are physiologically ready to breed.

Local weather conditions can also affect nest fate as it has been found for many birds, such as wild turkeys (*Meleagris gallopavo*) and greater sage-grouse (*Centrocercus urophasianus*) (Roberts and Porter, 1998; Webb *et al*., 2012), hence further affecting the fitness of a breeding population. Although a few studies have analysed the relationships between nest fate and weather conditions at the population level (Roberts and Porter, 1998; Dreitz *et al*., 2012; Roche *et al*., 2012; Webb *et al*., 2012), a global-level study on multiple species is lacking. Hence, we also need to study the relationship between weather and nest failure since weather can be a crucial element influencing the breeding success of a bird population.

Here, we studied 26 populations of plovers (*Charadrius* spp. and *Pluvialis dominica*) to examine the effects of the local weather conditions on breeding progression (changes in the number of nests/clutches laid across the breeding season) and nest fate in these birds. Plovers are ideal study organisms because they are globally distributed and breed on all continents except Antarctica, providing an excellent opportunity to find general trends in their breeding progression and nest failure across the globe (Piersma and Wiersma, 1996). Plovers are ground-nesting birds which breed in salt-pans, sea beaches, marshes and riverine habitats, and feed on insects such as dipterans, coleopterans and their larvae, shrimps, crabs, other crustaceans, worms, and molluscs (Johnsgard, 1981; Abensperg-Traun & Dickman, 1989; Székely *et al*., 1993; Fraga and Amat, 1996; Page *et al*., 2009; Pedro & Ramos, 2009). Multiple broods within a breeding season are common in southern breeders while northern breeders usually produce a single brood per season (Székely *et al*., 2008). Nest predation, flooding, strong winds, high tides, parental desertion and human disturbances are all well-known causes of nest failure in plovers (Page *et al*., 2009). Re-nesting by failed breeders commonly occurs 8 to 24 days after the loss of the previous clutch (Bergstrom, 1988; Fraga and Amat, 1996; Székely *et al*., 2008).

In this study, we first investigate how ambient temperature and precipitation were related to breeding progression, in terms of the number of nests laid by all members of a population collectively as the breeding season progresses. Second, we examine how the change in ambient temperature and precipitation was related to nest fate across populations. Addressing these questions together provides a deeper understanding of the effect of ambient weather conditions on the breeding progression and breeding success of shorebirds and also assist in predicting the effects of future climate change on this group of birds.

Materials and Methods

Study sites and fieldwork

We monitored nests belonging to 26 populations of 12 plover species (Charadriidae family) and one subspecies across the globe (Figure 1, Table S1.1 Supplementary material). Data were collected for one to ten years in the field depending on the study population (Table S1.1 Supplementary material). Nests were located by walking through appropriate habitat and watching for birds to flush off nests or by following birds back to their nests from a distance. Adults were captured at the nest using funnel traps or bow nets. The date of clutch completion or the date of clutch initiation were either known, for nests that were found during egg-laying and for nests with cameras; or estimated by floating eggs or measuring egg mass relative to egg size (Fraga and Amat, 1996; Liebezeit *et al*., 2007; Székely *et al*., 2008). For datasets where only the clutch completion date was recorded, we estimated the clutch initiation date by subtracting one day per egg laid, unless the time gap between successive eggs being laid was specifically known for the population, in which case that number of days was subtracted per egg in the clutch. Nest fate was recorded as failed or successful in the field based on direct observations such as flooding, predation, egg remains after predation, abandonment, etc.; or by using nest cameras to assess predation events. Nests were considered to have successfully hatched if one or more chicks were produced. Nests for which the fate was not known were removed from the nest failure analysis. Nests with predator exclosures or big nest cameras affect the fate of the nest by not allowing predators to reach the eggs and attracting predator attention or even scaring predators away, respectively, and hence were not included for any species (Tan *et al*., 2015; pers. comm. Dominic V. Cimiotti). Within sites, data for years with less than 10 nests recorded were excluded for the purpose of this study. Data from 21 populations was used for breeding progression analysis due to limited data availability of clutch initiation dates (Table S1.1 Supplementary material). Data for the nest failure study also belonged to 21 populations, with populations included in either or both analyses, since five populations did not have nest fate observations (Table S1.1 Supplementary material).

Data Collection

Breeding progression and nest failure

In this study, we investigated two variables in relation to weather conditions: breeding progression and nest failure. As a measure of breeding progression, we used clutch initiation dates to calculate the number of nests laid in a given week. Nest failure referred to the proportion of failed nests per day. It was calculated by taking the ratio of the number of nests failed to the number of nests both failed and hatched on a given day. We did not use an exposure-based model approach such as Mayfield or logistic exposure since we did not have the necessary information on number of exposure days from all the study populations. However, since our study's focus is not on population

level nest survival, our approach should suffice to study the effect of weather conditions on nest fate.

Weather data

Temperature and precipitation for the study period, of the site where each population was located, were used as proxies of the weather conditions. We downloaded high resolution historical daily weather data for the 40 nearest weather stations for each study site from the National Oceanic and Atmospheric Administration (NOAA) database, using the R package "*rnoaa*" (Chamberlain, 2017). For each study site, the data from the closest station with a complete record of daily temperature and precipitation for all the study years where bird data was available was then selected for analysis. The mean distance between the weather stations and the study sites was 55.59 ± 15.04 km (Supplementary Table S1.1). If the weather record was incomplete for any study site or if the weather station was more than 250 km away, we used the R package "*GSODR*" (Sparks *et al*., 2017) to extract weather data from the USA National Center for Environmental Information (NCEI) database. Hence, we used the data of the nearest weather station, the records of which optimally covers the study period of the given population. (see Table S1.1 for weather station details).

We derived four variables to characterise weather. To analyse breeding progression for each population, we averaged daily temperature (°C) and precipitation (mm) data over the pre-laying periods of one-, two- and three-weeks, before each week when nests were laid, in a moving window manner. That is, these two variables were calculated over three time frames for each week of the breeding season (period between first and last nest observations) in a given year and study site. We termed these as "pre-laying temperature" and "pre-laying precipitation" for one, two and three-weeks prior to egg laying week. A pre-laying interval of up to three weeks was selected since this reflects a common interval between the arrival of migratory species on the breeding grounds and the initiation of the first nests (pers. obs. Tamas Székely).

For testing associations between weather and nest failure we used a daily time scale that included average temperature (°C) and precipitation (mm) on each day up to seven days prior to nest fate, again in a moving window manner (Dinsmore *et al*., 2002; Dreitz *et al*., 2012). These were termed as "pre-fate temperature" and "pre-fate precipitation" for one to seven days prior to nest fate. Using the "scale" function in R (R Core Team, 2018), we z-standardised the weather variables for each year within each study site separately so that they have a mean of zero and SD of one. Therefore, our models do not include year as an explanatory variable.

Statistical Analyses

Breeding progression

We built generalised linear mixed models (GLMM) with binomial distribution of residuals to

investigate whether the breeding progression of plovers was affected by temperature and precipitation prior to egg laying. We used the proportion of nests laid per week for each year and site as the response variable, and the suite of "pre-laying temperature" and "pre-laying precipitation" variables from different time frames as explanatory variables in three different models. A non-linear effect was evident on visual inspection of the relationships (Figure S1.1, Supplementary material). Hence, quadratic terms for each of the explanatory variables were included. We added a nested random effect of population within species to the intercept to account for the relatedness between various populations and species at the subspecies level and also to account for the multiple observations within a site. We used data from 21 populations, and 9804 nests, collected across 1745 weeks spanning the period between 1988 and 2018 (Table S1.1 Supplementary material). These corresponded to eight species and two subspecies (*C. melodus circumcintus* & *C. nivosus nivosus*) of plover which was included to take into account the unresolved phylogeny and subspecies level differences. We used AIC values to compare the models from each analysis using different numbers of weeks for the pre-laying period to find which period's weather conditions best predicted the response. Models with lowest AIC values (within two AIC points) were considered the best models.

Nest failure

To analyse the effect of temperature and precipitation on the nest failure of plovers, we used data from 21 populations of plovers corresponding to 3602 days of 6917 nest observations collected between 1988 and 2019. These belonged to eight species and three subspecies (*C. melodus circumcintus, C. hiaticula hiaticula* & *C. nivosus nivosus*) of plover. We built GLMMs with a binomial distribution of residuals and modelled the proportion of failed nests per day for each year and each site as the response variable. We used "pre-fate temperature" and "pre-fate precipitation" as predictors. We included a random effect of species on the intercept instead of a nested random effect of population within species since that gave singularity warnings due to near zero variance being explained by part of the random effect. We built separate models with weather predictors from one to seven days prior to fate day and included quadratic terms for each. Quadratic terms were included as nest failure might increase on either side of optimum range of weather conditions due to increased energetic demands for parents to maintain the eggs at constant temperature and required water content levels. We used AIC values to compare the models from each analysis using different number of days as pre-fate period, to find which period's weather conditions best correlate with the response.

We conducted a Moran's I test which indicated no significant spatial autocorrelation in weather between the study sites (Moran's $I = -0.0038$, $p > 0.05$), and hence no spatial covariance term was included in the models in any of the analyses. We also checked for the correlation between the weather variables, used as predictor variables in our models, to avoid collinearity issues. The Pearson correlation coefficient was in the range of -0.09 to 0.01 for all covariates. We constructed

all models using the "*lme4*" package in R (Bates *et al*., 2015). We report results from a model as statistically significant if p equals or is smaller than 0.05.

To test for phylogenetic effects in our models, we constructed corresponding models using Bayesian Markov chain Monte Carlo generalised linear mixed models (MCMCglmm) approach using the "*MCMCglmm*" package in R (Hadfield, 2010). We followed the same method as that of Vincze *et al*. (2017) where we used a correlational structure of species-level phylogeny for each of the analyses (refer to Appendix 2 in Supplementary material for details). Due to extremely low phylogenetic signal, as has been found earlier in similar studies (Vincze *et al*., 2017; Halimubieke *et al*., 2020), we report results from the phylogenetic analyses in the supplementary material (Appendix 2). The phylogenetic structure is known for some (but not all) populations in our study and the maximum number of species in any analysis was only ten which is low for any phylogenetic analysis (see arguments in Losos, 2011; Cooper *et al*., 2016). Moreover, the phylogenetic models did not allow us to account for the nested effect of population within species and hence the GLMMs better represent the real case scenario. We used R version 3.5.2 (R Core Team, 2018) for all analyses and plotting.

Statement of animal ethics

This study did not involve any manipulation experiments, and all methods were carried out in accordance with relevant guidelines and regulations of each country in which it was performed. Fieldwork and bird-ringing procedures were authorized by relevant authorities: Hungary (Environmental Ministry and Kiskunság National Park); Australia (Deakin University Animal Welfare Committee Permits B02-2012, B20-2014 and B10-2016, State Government Permits 10006205, 10007918 and 10007241 and Australian Bird and Bat Banding Scheme (ABBBS) Authorities 1763, 3271 and 3033.); Canada: Canadian Wildlife Service Master Bander permit 10565 with endangered species endorsements, Environment Canada Animal Use Protocol Permits 2002-2006PNR016; Mexico (#SGPA/ DGVS/01717/10, #SGPA/DGVS/01367/11, #SGPA/DGVS/02078/12, #SGPA/DGVS/06485/13), Spain (Ministry of Environment #660117, and Consejería de Medio Ambiente, Junta de Andalucía and Xunta de Galicia); California (U.S. Fish and Wildlife (USFWS) #8035, #TE807078 and U. S. Geological Survey (USGS) #09316); Great Plains, USA (the U.S. Geological Survey Bird Banding Laboratory with Federal Master Bander permit #21446 with threatened and endangered species endorsements, Federal Threatened and Endangered Species handling permit#TE103272-3, and IACUC protocol #14-003.); Alaska (USGS Federal Master Bander permit #23269); China (Hebei Forestry Bureau); South Africa (Cape Nature and SAFRING); Cape Verde (Directorate Geral Ambiente); Turkey (Turkish Ministry of National Parks, Tuzla Municipality and Governor of Karatas, Mr. E. Karakaya); Portugal (Instituto da Conservação da Natureza e das Florestas, permit 01/2005-2018); New Jersey (Bird Banding Laboratory permit #23736, USFWS #TE35010D-0 and NJENSP permit #SC2019112, SUNY-ESF

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Results

Breeding progression and weather

The model with two-week weather averages was significantly better in predicting the odds ratio of the proportion of nests laid in the subsequent week as compared to the model with one and three-week weather averages (refer to Table S1.2 in Appendix 1 for AIC values for all models, and Table S1.3 and S1.4 for results from models with one and three-week weather averages). Results showed that two-week pre-laying period mean temperature and the proportion of nests being laid in the following week follow a quadratic relationship, such that the number of clutches laid per week was highest at intermediate temperatures (Figure 2A, see statistics in Table 1). Precipitation showed a linear relationship such that the proportion of nests laid slowly increases as the amount of precipitation increases (Figure 2B, Table 1).

Nest failure and weather

The model with average temperature and precipitation over three days before the fate day of the nest was the best predictor for the probability of nest failure (Table S1.5 shows AIC values for all models, Supplementary material). Temperature showed a quadratic relationship with the proportion of failed nests lowest at temperatures slightly higher than the mean (Figure 3A, Table 2). Average precipitation showed a linear relationship with proportion of failed nests increasing as the amount of precipitation increases (Figure 3B, Table 2).

Discussion

Plovers breed in relatively open sites and are hence exposed to the weather directly (Amat and Masero, 2004a) unlike many other birds which nest in cavities or tree canopies. This may make them more sensitive and vulnerable to changes in weather conditions compared to other bird groups. Hence, our study provides interesting insights regarding the effect of weather on plovers where the parents may be particularly constrained by the weather conditions. Our study has two key findings. First, average temperature and precipitation from two weeks prior to the laying week were associated with the number of nests that were laid in the given week. Second, average temperature and precipitation during the three days prior to nest fate were best associated with the probability of nest failure. We discuss each of these findings here in further detail.

Impact of temperature and precipitation on breeding progression

How the breeding season progressed for plovers (i.e., number of nests laid per week) was dependent on the average temperature and precipitation two weeks prior to laying. Several studies have shown that precipitation can predict breeding in many birds since it can lead to an increase in food abundance, especially insects and aquatic invertebrates (Immelman 1969; Sinclair, 1978; Hau, 2001; Batzer and Boix, 2016). Our results suggest that precipitation can be used not only as a cue for gonadal recrudescence in several bird species (Keast and Marshall, 1954; Immelman 1969; Halse and Jaensch, 1989), but also governs the laying decisions once the gonads are presumably ready. Our results show that the percentage of clutches laid in a given week shows a linear increase in relation to two week pre-laying period average precipitation. Plovers, like other birds, try to synchronise the hatching of chicks with food abundance peaks (Székely *et al*., 1993), which is often correlated with precipitation levels (Ali *et al*., 1977). Hence, it may be this synchronisation that leads to the observed pattern in our study. However, the effect may be very small as indicated by extremely low parameter estimates. Temperature, on the other hand, plays a significant role in governing the number of clutches being initiated in a given week. We found that the number of nests initiated in a given week is the highest at an optimum range of temperature, on either side of which it decreases. This observation may have two possible explanations.

First, the adult birds may avoid extreme hot or cold temperatures to reduce stress of maintaining viable egg temperatures. A number of studies have demonstrated that along with the food requirements of chicks, the energetic requirements of parents are important factors governing the timing of breeding in birds (Perrins, 1991; Tulp *et al*., 2009). The energetic requirements for parents may vary between different periods of breeding such as pre-laying, incubation and brood-rearing (Tulp *et al*., 2009). Plovers are known to breed across large geographic scales and can experience extreme temperatures even during the breeding season, thus potentially facing constraints on their time and energy budgets (Grant, 1982; Amat and Masero, 2004b; Tulp *et al*., 2009). In several places, such as in the Southern High Plains of Texas and southern Spain, they may experience very high temperatures during egg laying and are also known to avoid shaded areas for nesting as an anti-predator strategy which further necessitates thermoregulatory behaviours such as behavioural shading of eggs and cooling off by parents themselves (Grant, 1982; Amat and Masero, 2004a; Amat and Masero, 2009; Page *et al*., 2009; Saalfeld *et al*., 2012; Lomas *et al*., 2014). Plovers exhibit various behaviours, such as back-rest and belly-soaking, that involve dissipating body heat for the parents and cooling the eggs at extreme hot temperatures (Grant, 1982; Amat and Masero, 2007; Page *et al*., 2009). Other plover species breeding in the high arctic, face very low temperatures, and need incubation to maintain the eggs at a constant temperature (Piersma *et al*., 2003; Tulp *et al*., 2009). However, these behaviours can be costly in terms of energy and time for these birds, and also leave less time for foraging by the parents for food uptake to cope with higher energetic demands (Grant, 1982; Hinsely and Ferns, 1994; Piersma *et al*., 2003; Tulp *et al*., 2009). This may be one reason why the number of clutches initiated per week were significantly lower at extremely

low or high temperatures. The parents may monitor the ambient temperature for a period of about two weeks which might entail the mating and egg formation period after which the decision to lay eggs may be taken. Hence, average temperature over two weeks may be a better predictor as compared to averages over one or three weeks prior to the laying week. However, one should note that not every population or species may be the same and these results indicate a general trend on a global scale. The American golden-plover (*Pluvialis dominica*) should be noted as an exception in this study since it nests in the Arctic tundra and most birds arrive and start laying within a week or less. Hence, the model with one week prior weather variables may be more valid for such populations although the patterns remain the same across all models. The difference in AIC values was also very low between models with one and two week prior weather averages.

Second, the two week period of mating and egg formation, known as the pre-laying period, may also be very energetically demanding for the parents (Tulp *et al*., 2009). Since food availability for adult plovers can be affected by environmental conditions such as temperature (Ali *et al*., 1977; Pienkowski, 1983; Batzer and Boix, 2016), the parents may not have enough food available at extreme temperatures to fulfil the energetic requirements of egg laying or nest formation. This can therefore result in lower number of clutches being laid in the subsequent week, hence explaining the observed pattern in our study of most clutches being laid at a certain range of temperature on either sides of which the number goes down.

Impact of temperature and precipitation on nest failure

The probability of nest failure was most tightly associated with the average temperature and precipitation in the three days prior to the failure or hatching of the eggs. The models with one, two and four days prior weather conditions also showed similar patterns but the AIC value was lowest for the model with three day weather averages. This may indicate the threshold period for embryonic development to stop if favourable conditions cease to exist. However, it can also be an artefact of field observations since it is nearly impossible to know the exact date of the nest fate in the field unless the nests are monitored daily or have nest cameras installed. Since many populations had nest cameras and in other cases the data was included only if the fate date was known with given certainty, the results should depict real correlations.

Plovers are ground-nesting birds which mostly build nests in small cup-like depressions in the ground which can easily accumulate water and hence lead to nest failure due to flooding which is most often caused by rain and hail (Bergstrom, 1988; Conway *et al*., 2005; Page *et al*., 2009; Saalfeld *et al*., 2011; AlRashidi *et al*., 2012; Roche *et al*., 2012). Increased precipitation may also result in conditions of higher ambient water vapour content which can inhibit water vapour loss from the eggs, an integral part of egg development (Grant, 1982; Rahn, 1984; Walsberg and Schmidt, 1992). Hence, an increase in precipitation is expected to lead to nest failure through two processes. Previous studies on Mountain plovers (*Charadrius montanus*) and other birds have found

precipitation to negatively affect daily nest survival (Dinsmore *et al*., 2002; Dreitz *et al*., 2012; Webb *et al*., 2012). A study on Greater sage-grouse (*Centrocercus urophasianus*) established a significant association between a one-day lag in precipitation and nest survival (Webb *et al*., 2012). Our study showed similar results where precipitation with three day lag significantly predicted the proportion of nests failed. The proportion of nests failed increases linearly as precipitation increases and reaches almost hundred percent at very high precipitation levels above the mean. This may be attributed to causes listed above, namely, nest flooding and reduced water vapour loss from the eggs. Average precipitation over one to five days prior also shows similar relation to nest failure. However, inhibition of water vapour loss for three continuous days may be the threshold for the plover eggs and hence shows up as the most well correlated period in our results. A study on daily nest survival of mountain plovers previously found a small significant effect of a ten-day drought period (≤ 1 mm precipitation for ten or more days) (Dreitz *et al*., 2012). However, in our study, precipitation did not have any effect beyond six days prior to nest fate. These differences between studies may be attributed to differences between species and populations. Since our study combined several populations and species of plovers, we see associations between nest fate and precipitation similar to other studies. The associations are significant with precipitation over one to five days prior to nest failure which may depict the general global trend for the family Charadridae.

Besides effects of precipitation, temperature also influences the probability of nest failure in plovers as shown by results in our study and previous studies on some plover populations (Dreitz *et al*., 2012). The probability of nest failure is lowest at an optimum range of three day pre-fate day period average temperature, on either side of which it increases. This optimum range of temperature occurs at slightly higher than the average temperature levels for the study sites. It is known that avian eggs have an optimum range of temperature for development and survival, which varies for different groups and species (Webb, 1987). Lethal high temperatures for eggs, high temperatures at which the eggs fail to hatch, vary across bird species and are also known to occur across some of our study sites (Bennett *et al*., 1981; Grant, 1982; Amat and Masero, 2004a; Saalfeld *et al*., 2012). However, eggs can survive such lethal temperatures for very short durations of less than an hour but have to be incubated or shaded for most of the time (Grant, 1982; Saalfeld, 2010). As mentioned before, such activities increase the daily energy expenditure and are time-consuming, hence leaving the parents with little time to forage (Grant, 1982; Hinsley and Ferns, 1994). Such lethal egg temperatures, which would vary across species and study sites, would occur during hot weather conditions that also imply stressful conditions for the parent birds that need to resort to behavioural thermoregulation which can include behaviours such as belly-soaking and back-rest (Grant, 1982; Amat and Masero, 2007; Ryeland *et al*., 2017).

Plovers exhibit extensive incubation behaviours to keep the eggs at a constant temperature in extreme environments. However, it may be particularly difficult for these birds to strike a balance between incubation (warming) or shading (cooling) of eggs, and self-maintenance behaviours such

as dissipating heat or foraging in such extreme weather conditions (Grant, 1982; Amat and Masero 2004b). Lack of parental attendance to eggs then may result in reduced hatching success and desertion of broods in some species. Amat and Masero (2004b) showed that Kentish plover (*Charadrius alexandrinus*) males increased their participation in diurnal incubation at high ambient temperatures, and also that during such conditions pair members resorted to shortening incubation bouts. However, during heat waves the plovers were unable to attend their nests continuously, and nests were deserted. The propensity of the plovers to desert their nests was affected by proximity to water, indicating that water proximity changed adults susceptibility to thermal stress through the possibility of belly soaking.

In parrotlets, exposure of three or more days can lead to reduced hatching success of eggs even at normal temperatures (Stoleson and Beissinger, 1999). If the plover parents leave the nests unattended for long or even several short bouts at extreme temperature conditions, perhaps over a period of three days, it might lead to failure of embryonic development. This could happen by either crossing the upper lethal temperatures for eggs several times, or even periodic cooling of eggs which has been found to have an effect on embryonic growth efficiency in zebra finches (*Taenopygia guttata*) (Olson *et al*., 2006). Thus, it could explain the observed trend in our study. However, the above-mentioned study involved captive zebra finches which are passerines and hence are quite different and of limited relevance to our study. However, similar studies on plovers in the future could help test some of these ideas in the non-passerines as well.

Our results also show that temperatures, lower than the average, affected nest failure more than temperatures higher than the average experienced by the birds at a given site. This implies that it may be more challenging for adult plovers to maintain constant temperatures for eggs when the ambient temperatures are lower than the average. To summarise, at lower temperatures it may be challenging to maintain egg temperature, but at high temperatures it may be challenging to maintain body temperature of incubating adults. Since plovers usually nest in exposed sites where incubating adults receive direct solar radiation, it makes incubating birds particularly susceptible to thermal stress at high ambient temperatures (Amat and Masero, 2004a; Amat and Masero, 2009; Page *et al*., 2009; Saalfeld *et al*., 2012; Lomas *et al*., 2014).

The high stress for incubating birds at extreme temperatures leading to shorter bouts of incubation due to the parents leaving for behavioural thermoregulation more often, suggests that eggs are left unattended for longer periods (Amat and Masero, 2004b). This could also lead to higher nest predation in such conditions due to unattended nests not being actively protected by the parent birds. Nest predation, apart from weather, is a leading cause of nest failure in plovers and the intensity often varies throughout the breeding season (Page *et al*., 1983; Pienkowski, 1984; Bergstrom, 1988; Melvin *et al*., 1992; Fraga and Amat, 1996; Page *et al*. 2009; Saalfeld *et al*., 2011). Nest predators may vary between species and habitats but some of them include foxes, domestic dogs, cats, lizards, snakes, corvids, terns and gulls (Fraga and Amat, 1996; Page *et al*., 2009).

Our work highlights the importance of studying the effects of environmental variables on breeding decisions even after the start of the breeding period since just a shift in the initial or mean laying date is not sufficient to judge the breeding success of a population in cases of sudden changes in weather conditions, which may be exacerbated due to climate change. Further, this study also sheds light on some environmental variables that may be used as cues by birds or be guiding re-nesting decisions after previous clutch failures through effects on other factors. Our results help describe some of the mechanisms through which stochastic weather changes could impact breeding success of ground-nesting shorebird populations across the world. This is essential to study since climate change is expected to increase the frequency of stochastic events in most places which can then affect the bird populations (eg: Ojima and Lackett, 2002; Dreitz *et al*., 2012). Therefore, similar research avenues should be explored for other bird groups as well which may be affected by such stochastic changes in the weather conditions. Since this is the first study of its kind examining the effect of weather on breeding after the start of the breeding season, and in which the role of environmental factors on nesting initiation in birds has been investigated at a scale of within and across breeding seasons, it had certain limitations. For example, we studied the effects of temperature and precipitation individually but they may act together in the natural environment. Also, we set out to examine global general trends across populations which may have left out relationships present at individual sites. However, understanding the role of individual variables is a step towards understanding more complex interactions in nature. Future work could also include effects of other environmental variables such as tides and wind speed.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1 Breeding progression, expressed as proportion of nests laid each week (logit scale), in relation to ambient temperature and precipitation two weeks prior to egg-laying. Data corresponds to 9803 nests belonging to 21 populations of plovers, collected across 1745 weeks. N = 1745. Significant p-values ($p < 0.05$) are marked in bold.[1]

Fixed effects	**Estimate**	**Standard error**	***z* value**	***p* value**
Intercept	-2.28	0.18	-12.34	**<0.01**
Temperature average	0.17	0.02	7.69	**<0.01**
Temperature average^2	-0.36	0.02	-15.56	**<0.01**
Precipitation average	0.04	0.02	2.45	**0.01**
Precipitation average^2	0.01	0.01	1.02	0.31

Random effects	**Variance**	**Standard deviation**
Population:species	0.04	0.21
Species	0.30	0.55

[1] The temperature and precipitation averages given in this table belong to the two-weeks prior to the laying week since they were the best predictors of breeding progression. Similar analyses were done for weather averages from one and three-weeks prior to the laying week (refer to Table S1.3 & S1.4 in Appendix 1 for results from those models).

Table 2 Nest failure, expressed as proportion nests failed each day (logit scale), in relation to pre-fate temperature and precipitation three days prior to fate day of nest. Data corresponds to 7074 nests belonging to 21 populations of plovers, collected across 3699 days. N = 3699. Significant p-values ($p < 0.05$) are marked in bold.[1]

Fixed effects	Estimate	Standard error	*z* value	*p* value
Intercept	0.29	0.18	1.65	0.10
Temperature average	-0.35	0.04	-8.46	**<0.01**
Temperature average^2	0.02	0.01	3.35	**<0.01**
Precipitation average	0.11	0.03	3.62	**<0.01**
Precipitation average^2	0.00	0.00	0.19	0.85

Random effects	Variance	Standard deviation
Species	0.30	0.55

[1] The temperature and precipitation averages given in this table belong to the three-days prior to the fate day of the nest since they were the best predictors of nest failure. Similar analysis was done for weather averages from one to seven days prior to the fate day (refer to Tables S1.6-S1.11 in Appendix 1 for results from those models).

Figure legends

Figure 1 Study site locations and species / subspecies populations (N = 26) used in an analysis to investigate ambient temperature and precipitation on plover breeding progression and nest survival. (Copyright for species illustrations: pending)

Figure 2 Predicted marginal effects of temperature and precipitation on the percentage of nests laid per week. (a) shows the significant parabolic effect of increasing temperature average of *three weeks prior* to the laying week on the percentage of nests laid per week. (b) shows the significant parabolic effect of increasing precipitation on the percentage of nests laid per week. The x-axis parameters have been z-standardized, such that values towards the left represent lower temperature and precipitation, and values on the right represent higher temperature and precipitation. The shaded area represents the 95% confidence interval. Data used belongs to 21 plover populations from eight different species and one subspecies recorded across 1745 weeks for 9803 nests.

Figure 3 Predicted marginal effects of temperature from *three days prior* to fate day on the percentage of failed nests per day in each population. (a) shows the significant linear effect of increasing average precipitation on the percentage of failed nests. (b) shows the significant parabolic effect of increasing temperature average on the percentage of failed nests. The x-axis parameters have been z-standardized, such that values towards the left represent lower temperature and precipitation, and values on the right represent higher temperature and precipitation. The shaded area represents the 95% confidence interval. Data includes 7074 nests from 21 plover populations from ten different species and one subspecies collected over 3699 days.

Figures

Figure 1

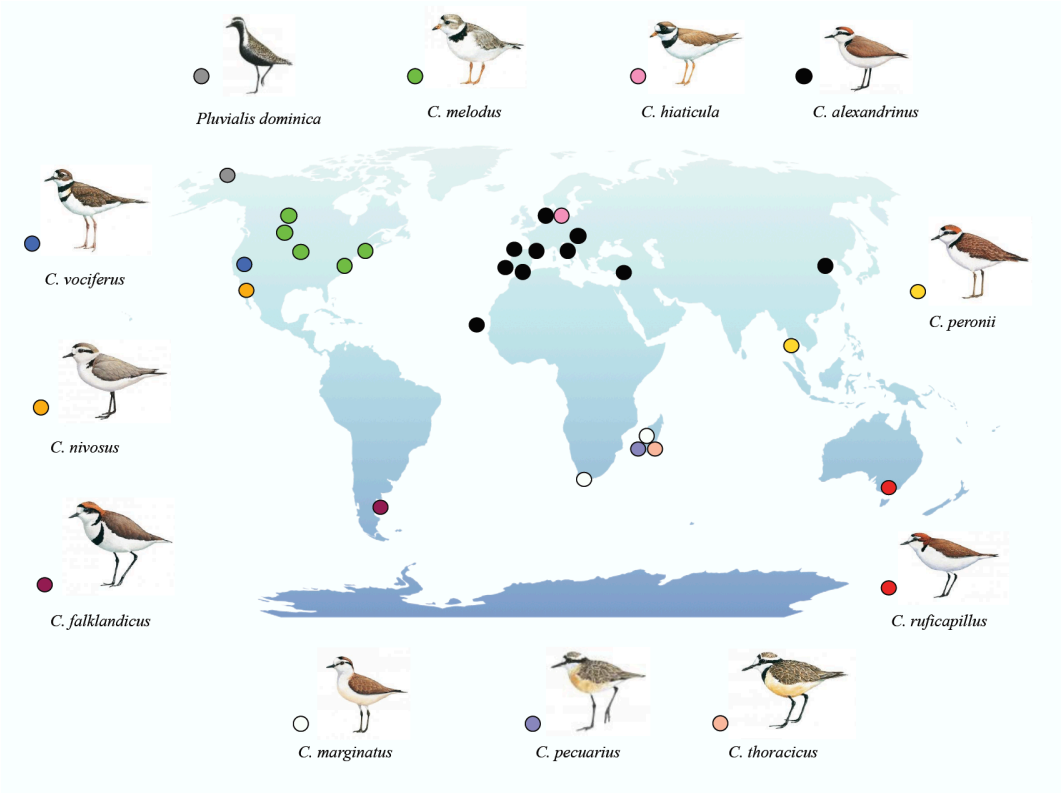


Figure 2

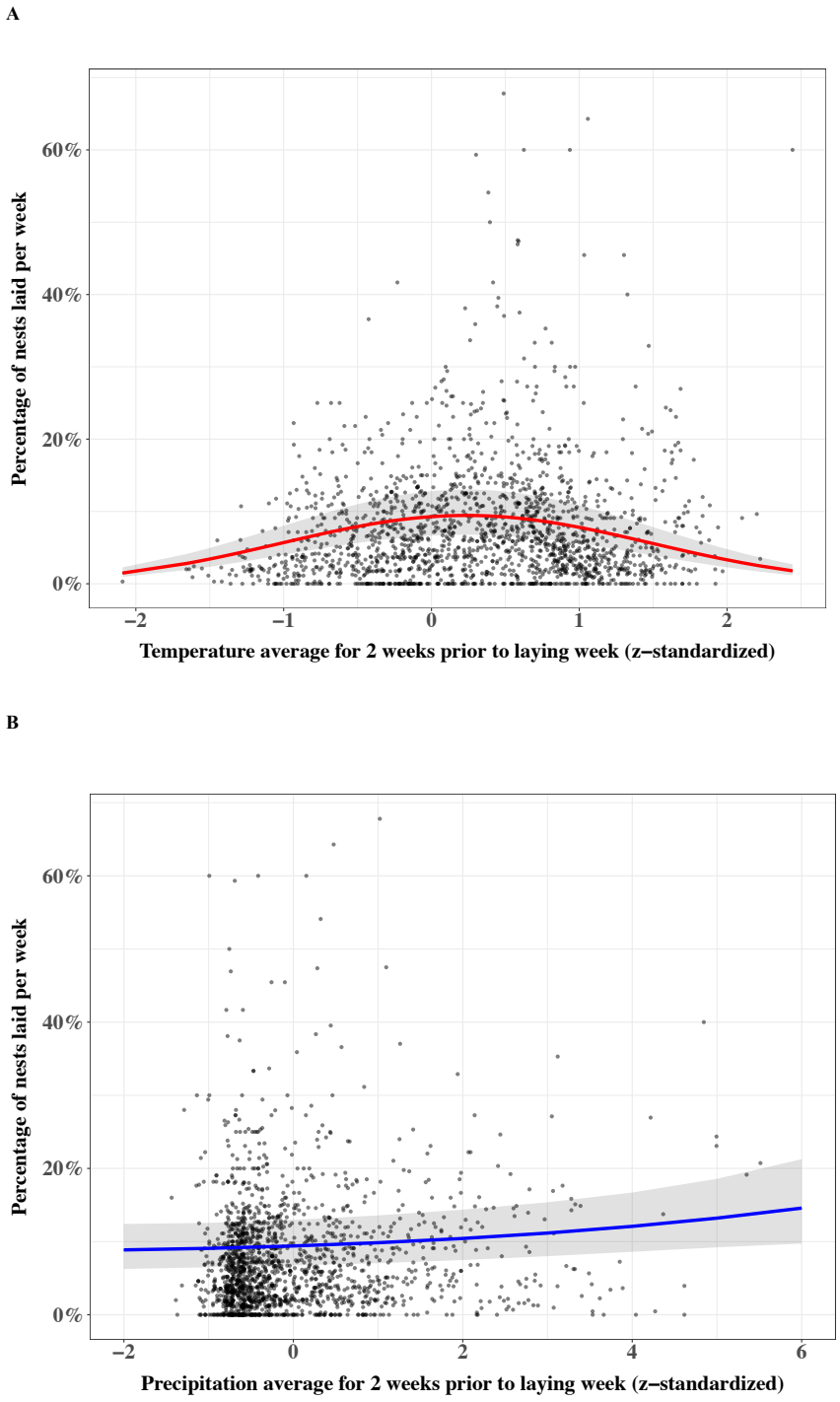
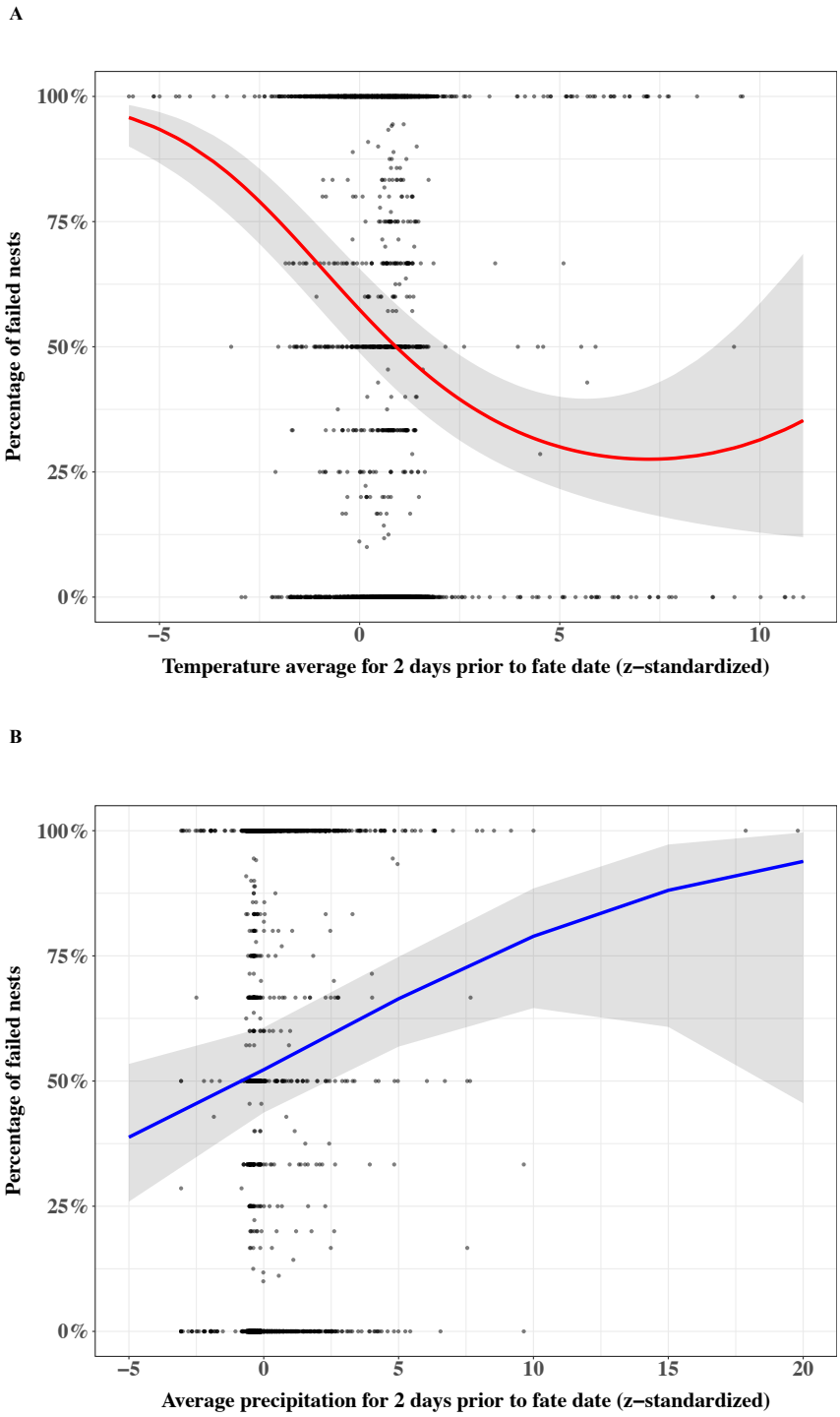

A
Percentage of nests laid per week
60%
40%
20%
0%
−2
−1
0
1
2
Temperature average for 2 weeks prior to laying week (z−standardized)
B
Percentage of nests laid per week
60%
40%
20%
0%
−2
0
2
4
6
Precipitation average for 2 weeks prior to laying week (z−standardized)

Figure 3


A
100%
75%
50%
25%
0%
Percentage of failed nests
−5
0
5
10
Temperature average for 2 days prior to fate date (z−standardized)
B
100%
75%
50%
25%
0%
Percentage of failed nests
−5
0
5
10
15
20
Average precipitation for 2 days prior to fate date (z−standardized)

Appendix IV | Mortality cost of sex-specific parasitism in wild bird populations

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Manuscript in review in Scientific Reports

Authors' Contributions

JOV and TS conceived and designed the research;
JOV conducted the data analysis and wrote the paper;
AL and TS provided part of the mortality and life history data;
all authors contributed substantially to revisions of the paper and gave final approval for publication.

Abstract

Sex-specific mortality is frequent in animals although the causes of different male versus female mortalities remain poorly understood. Parasitism is ubiquitous in nature with widespread detrimental effects to hosts, making parasitism a likely predictor of sex-specific mortalities. Using sex-specific blood and gastrointestinal parasite prevalence from 96 and 54 avian host species, respectively, we test the implications of parasites for annual mortality in wild bird populations using phylogenetic comparative methods. First, we show that parasite prevalence is not different between adult males and females, although Nematodes showed a statistically significant but small male-biased parasite prevalence. Second, we found no correlation between sex-biased host mortalities and sex-biased parasite prevalence. These results were consistent in both blood and gastrointestinal parasites. Taken together, our results show little evidence for sex-dependent parasite prevalence in adults in wild bird populations, and suggest that parasite prevalence is an unlikely predictor of sex difference in adult mortalities, not withstanding sampling limitations. We propose that to understand causes of sex-biased mortalities, more complex analyses are needed that incorporate various ecological and life history components of animals life that may include sex differences in exposure to predators, immune capacity and cost of reproduction.

Keywords

comparative method, meta-analysis, sex ratio, sexual size dimorphism, body size, survival

Introduction

Although sex ratio at birth is often close to 1:1 in wild populations, adult (or tertiary) sex ratios (the proportion of males to females in a population) are highly variable suggesting that sex differences in post-birth maturation, mortalities and/or population movements drive skewed adult sex ratios (ASR) (Wilson 2000, Le Galliard *et al*. 2005, Székely *et al*. 2014b, Ancona *et al*. 2017).

Mortality is a complex process, influenced by many factors that in simple terms could be classified as intrinsic and extrinsic to the individual (for example, immune capacity and ambient environment, respectively, Williams *et al*. 2006). Predation, disease and starvation are important causes of mortality in wild animal populations, whereas body size and sexual selection are general predictors of mortality according to life history theory (Calder 1984, Boukal *et al*. 2008), with larger animals often dying at lower rates than smaller ones (Pauly 1980, Healy *et al*. 2014, Valcu *et al*. 2014, Scharf *et al*. 2015). Furthermore, social activities such as competition for food and/or mates may increase mortality of one sex more than the other (Darwin 1871, Liker and Székely 2005, Jennions and Kokko 2010).

One important cause of mortality are pathogens or infectious agents. For instance, if we consider the history of the modern human, it has been marked by diseases of epidemic scale that resulted in millions of deaths that were caused by bacteria, viruses and parasites (Hays 2005). Whereas in wild animals, extreme examples include native species exposed to exotic pathogens, driving populations to critically low numbers (e.g. Serengeti's wild dogs and lions, Roelke-Parker *et al*. 1996, Darwin's finches, Kleindorfer *et al*. 2014), or even to the edge of extinction (Wyatt *et al*. 2008). Moreover, pathogens have shown to also provoke mortality not by directly killing but debilitating and deteriorating the overall condition of the prey, increasing the chances of predation (Møller and Erritzøe 2000, Genovart *et al*. 2010, Ingram *et al*. 2013, Adelman *et al*. 2017).

Interestingly, despite the undoubtable relationship between pathogens and mortality in animals, information on the relationship between sex-biased infections and biased sex ratio is scarce. A notable exception occurred in mammals, where Moore and Wilson (2002) found a positive correlation across 106 mammal species for the bias in sexual size dimorphism (SSD) and the sex bias in parasitism, and that sex bias in parasitism predicted the sex bias in mortality, concluding that sexual selection for the larger sex (i.e. males) implicated a mortality cost through parasitism (see also Promislow *et al*. 1992). Also, male mammals have a weaker immune competence, which correlates with higher presence of pathogens and mortality compared to females (Klein and Roberts 2010, but see Kelly *et al*. 2018). In birds, sex-biased infections and its implications on survival have not been assessed across a broad range of taxa although indirect evidence would suggest so since a previous across-species meta-analysis found a male-biased prevalence of gastrointestinal parasites (Poulin 1996) in addition to more recent complementary evidence showing that larger avian species are more affected by parasites, possibly because in larger hosts, parasites have greater space and niches to colonize and are likely to accumulate through life as larger species tend to live longer than

smaller ones (Healy *et al*. 2014, Kamiya *et al*. 2014, Gutiérrez *et al*. 2017). Therefore, from this perspective and considering that in birds males are in general larger than females (Székely *et al*. 2007), we could expect parasitism in birds to be male-biased. Although this difference in size is modest compared to mammals, and it is unknown at what extent this could influence parasite load between the sexes. From a hormonal perspective, the sex hormones influence the reproductive behavior e.g. courting, territoriality, aggression, competition and nesting (Folstad and Karter 1992, Adkins-Regan 2005), which could translate into sex-different rates of parasite acquisition due to immunosuppression mediated by testosterone or stress-released corticosterone (cost for males, Duffy *et al*. 2000, Klein and Roberts 2010, Gao *et al*. 2017), or sex-differences in behavior such as nesting time or breeding dispersal (cost for females, Korpimäki *et al*. 1993, Végvári *et al*. 2018). However, current studies disregard the effect of sex hormones in immunity, as well as challenge the idea of sex-different immune response in birds, as shown by Roberts *et al*. (2004) and more recently by Foo *et al*. (2017), finding little evidence supporting a significant effect of sex hormones in immunity. Moreover, Kelly *et al*. (2018) showed a lack of sex-differences in immunity across 241 immune estimates in birds, and Peters *et al*. (2019) showed that, in general, immunosenescence also lacked sexual dimorphism across animals, including birds.

Thus, the current evidence give males a small advantage over females as the sex more likely to be affected by parasites in birds (Poulin 1996, Kamiya *et al*. 2014, Gao *et al*. 2017), but is nevertheless unknown whether this variable could relate to the overall lower male mortality compared to females found in birds by Liker and Székely (2005) and suggested by their overall male-biased ASR (Donald 2007, Székely *et al*. 2014b).

Nevertheless, studies using unsexed birds suggest an association between mortality and both gastrointestinal and blood parasitism, supported by evidence established through direct analysis of carcasses of mortality events or through capture-recapture survival analyses (e.g. Spalding *et al*. 1993, Martínez-de la Puente *et al*. 2010, Robinson *et al*. 2010, Lachish *et al*. 2011).

To examine the relationship between sex-specific parasite prevalence and mortality in birds, we obtained data from a total of 138 bird species (across 96 species from 13 avian orders for blood parasites and 54 species from 9 orders for gastrointestinal parasites) from published literature to test two hypotheses using phylogenetic comparative analyses. First, we investigate whether males had higher parasite prevalence compared to females, as predicted by male's modest but significantly larger body size (Székely *et al*. 2007), male's frequent stress-inducing behavior (corticosterone mediated immunosuppression, Adkins-Regan 2005, Gao *et al*. 2017), and as previously shown in across-species studies in mammals (Moore and Wilson 2002) and in birds (particularly gastrointestinal parasites, Poulin 1996). Second, we evaluated whether sex-specific parasite prevalence predicted sex-specific adult annual mortality. Specifically, we (i) tested the effect of parasite prevalence on mortality on males and females separately as they present variation in their physiology and life histories (Immonen *et al*. 2018) that could influence the degree of exposure

and/or infection to parasites and subsequent mortality (Maklakov and Lummaa 2013, Tarka *et al*. 2018). Then, we (ii) tested whether sex-specific adult annual mortality was predicted by sex-specific adult parasite prevalence, including SSD and mating competition in the analysis as potential confounding variables (Sæther 1989, Promislow *et al*. 1992, Székely *et al*. 2014a).

Materials and methods

Literature search

We collected data of sex-specific prevalence of parasitism in birds using ISI Web of Science and Google Scholar, by using the following keyword combinations: "scientific name of host species" + parasit*, prevale*, helmint*, blood, malar*, haemoparasit*, mite* or lice. The list of names searched initially corresponded to 369 bird species included in the dataset of sex-specific annual mortality data provided by Székely *et al*. (2014a). If the bird species name had synonyms, the search was repeated with every name. The references of previous reviews and meta-analyses were also checked. The inclusion criteria required the parasite prevalence to be: (i) determined from adult birds with known sex, (ii) obtained from wild birds (not captive), and (iii) from infection naturally acquired (not experimentally infected). We only included studies reporting results for both males and females to avoid difficulties comparing prevalences within species generated by different sampling/diagnostic methods or different populations. We included studies with haemoparasite detection through molecular and optic microscopy methods because both bring comparable results and to date there is not consensus about which technique is better over the other (Valkiūnas *et al*. 2008, Ishtiaq *et al*. 2017). All studies available for gastrointestinal and external parasites used exclusively taxonomic keys diagnosis through microscopic examination. Studies based on parasite's egg counts were not considered to minimize the chances of including studies containing false negative results originated by the variation in egg shedding rhythms seen in some gastrointestinal parasites (López *et al*. 2007). In order to obtain a robust estimate of parasite prevalence for a given host species, all publications that met the inclusion criteria were included in our dataset.

Body mass, adult mortality and sexual competition

Data on sex-specific body mass, annual adult mortality and sexual competition were obtained from Székely *et al*. (2014a). Data was augmented following the method provided by Székely *et al*. (2014a) and Liker *et al*. (2014), consisting of searching the name of the additional bird species in scientific citation indexes, books, species monographs and electronic databases. We included mortality estimates obtained from field studies in which the estimate for both males and females were determined in the same population and with the same method. Three main methods were used to determine mortality rates: capture-recapture, ringing recoveries and local return rates. Mating system was determined as a five-point score of the frequency of polygamy for each sex, with "0"

corresponding to very rare or no polygamy, "1" to rare polygamy, "2" to uncommon polygamy, "3" to moderate polygamy and "4" to common polygamy (for more details see Liker *et al*. 2014).

Parasite prevalence

The final dataset included 96 bird species (sample size range of 4–1045) with sex-specific blood parasite prevalence data, 54 species (5–9729) with gastrointestinal parasite prevalence data and only 3 species (13–131) with ectoparasite prevalence data. Ectoparasites were excluded from further analyses due to the low sample size. Blood parasites were divided into five categories: *Haemoproteus*, *Leucocytozoon*, *Plasmodium*, *Trypanosoma* and Microfilaria. Whereas gastrointestinal parasites were categorized as Cestoda, Acanthocephala, Nematoda, Trematoda and Protozoa. Finally, one last category received data presented as the combination of two or more parasite categories (for example, we often found blood parasite studies reporting the overall prevalence of *Haemoproteus*, *Leucocytozoon* and *Plasmodium*, three parasites categories combined in one single datum). Some studies of blood parasitism included avian species that presented 0% prevalence in both sexes. These studies were included in the dataset, although it was uncertain whether birds never got parasitized due to vector absence in their habitats (Piersma 1997), were able to reduce parasitemia under detection limits, or because the parasites were unable to complete their life-cycle in the host (Martínez-Abraín *et al*. 2004). Along with parasite prevalence data we also recorded the period of the year when parasites were samples, which was divided in three categories: breeding (sampling took place mostly during the hosts' breeding period), nonbreeding (sampling took place outside the breeding period) and year-round (sampling included both breeding and nonbreeding periods).

Phylogenetic meta-analysis

To investigate sex difference in parasite prevalence, a phylogenetic multivariate meta-analysis was performed using the R package metafor (Viechtbauer 2010). Because all studies only provided prevalence and sample size values, we opted to group the birds as infected and not infected males and females in 2 x 2 contingency tables and then calculate the effect size as odds ratio (Viechtbauer 2010). We conducted multivariate random-effect meta-analyses including period of sample (breeding, nonbreeding and year-round) and method of parasite detection (only for blood parasites, consisting of three categories: molecular detection, optic microscopy detection, and both) as moderators (i.e. fixed-effect), and study and phylogeny (a variance-covariance matrix) as random-effect variables.

Publication bias (due to missing studies that were not published because of negative or null results, Koricheva *et al*. 2013) was evaluated using Egger's regression test (Egger *et al*. 1997, Sterne and Egger 2005) by including the standard error of the effect sizes as an additional moderator within the model. If the intercept significantly deviated from zero (significance of $P < 0.10$, Egger *et al*.

1997) the overall relationship between the precision and size of studies included in the dataset was considered asymmetrical, or in other words, biased (Sterne and Egger 2005). Of the twelve models conducted, two suggested presence of publication bias, corresponding to the gastrointestinal parasites Nematoda (P = 0.035) and Trematoda (P = 0.043). Diagnostic tests for identifying influential data points and outliers, and rules for excluding these types of cases are still evolving, particularly for multivariate/multi-level meta-analytical models (Viechtbauer and Cheung 2010). To address this we used the approach described by Habeck and Schultz (2015), consisting of identifying the influential outliers causing the bias and running the models after excluding these values.

Statistical power in random-effects meta-analysis can be difficult to determine. Jackson and Turner (2017) showed that, in general, meta-analyses with at least five studies offer more power than the individual studies alone. Therefore outcomes below this five-studies threshold should be taken carefully.

Phylogenetic comparative analysis

We used phylogenetic generalized least squares (PGLS) to test whether parasite prevalence was related to annual mortality, adult body mass and sexual competition. This approach allows controlling for the non-independence among species by incorporating a variance-covariance matrix that represents their phylogenetic relatedness (Symonds and Blomberg 2014). In all models we used Pagel's lambda (λ) as measure of phylogenetic signal (Molina-Venegas and Rodriguez 2017) and it was set to the maximum-likelihood value (Freckleton *et al*. 2002). Prior to the analyses, prevalence and mortality were arcsine-square-root-transformed. Mortality bias was expressed as log(male mortality/female mortality). Average body mass (in grams) of male and female adults was log-transformed, whereas SSD in adult body mass was expressed as log(male body mass (g)/female body mass (g)). The sex bias in mating system was calculated as the difference between male and female polygamy scores (Liker *et al*. 2014). Because each host species had several estimates of prevalence, the sex bias in parasite prevalence of each bird species was incorporated into this analysis as the weighted average effect size of all comparisons. Thus, for each individual host species, we obtained a single value representative of all parasite infections measured in different studies. The effect size per species was calculated using the function *escalc* of the R package *metafor* with odds ratio as measure. We fitted both single-predictor and multi-predictor models to blood parasites and gastrointestinal parasites and each model was run separately for females, males and sex bias. To account for phylogeny, we used the avian phylogeny from Jetz *et al*. (2012). The analyses were run using consensus trees (one for each type of parasitism, **Fig. S3.1**) obtained through the method 50% majority-rule (Holder *et al*. 2008, Sukumaran and Holder 2010) from 1,000 randomly selected trees from a pool of 10,000 available (http://birdtree.org), using the methodology described by Rubolini *et al*. (2015). These phylogenetic trees were not fully resolved,

and polytomies were arbitrarily resolved by adding a branch distance of 10^{-08} to one randomly chosen branch in the polytomy using the function *multi2di* from the R package *ape* (Paradis *et al*. 2004). All PGLS analyses were conducted in R using the package *caper* (Orme *et al*. 2013).

Results

Phylogenetic meta-analysis

Overall, males and females did not exhibit different prevalence of blood parasites nor gastrointestinal parasites (**Fig. 3.1** and **Table 3.1**). In the analysis broken down for parasite category (five categories of blood parasites and five of gastrointestinal parasites; **Table 3.1**), only Nematodes showed a weak male-biased prevalence (**Fig. 3.1b**; k = 33, estimate = 0.388, Z statistic = 1.979, P = 0.048, 95% CI = 0.004, 0.773).

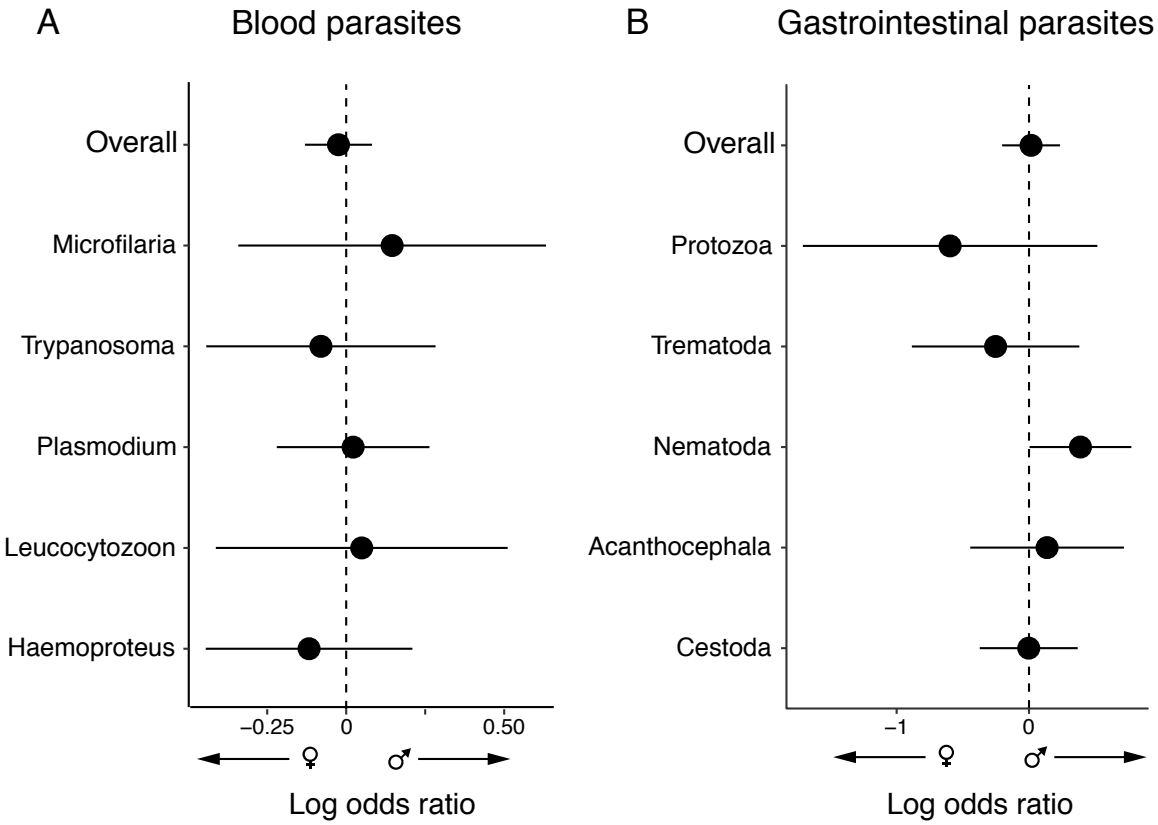

Figure 3.1 Sex bias in prevalence of (A) blood parasites and (B) gastrointestinal parasites in birds. Weighted average effect size estimates, showing lower and upper 95% confidence intervals in overall meta-analyses and broken down results according to parasite category (see Material and methods). The dashed vertical line indicates no sex difference, positive values represent male bias prevalence and negative values female bias. See **Table 3.1** for statistics.

Parasite prevalence and annual adult mortality

We found no association between annual mortality and prevalence in either blood parasites or gastrointestinal parasites (**Table 3.2**). The lack of association was consistent when each sex was tested separately (**Table 3.2**) and also when analyzing the sex bias (**Fig. 3.2** and **Table 3.2**).

These results remained qualitatively unchanged after conducting multi-predictor analyses incorporating body mass and mating competition into the models (**Table 3.2**). In these latter analyses only body mass and mating competition had a significant effect on mortality, although the relationship with mating competition was significant only in the blood parasite analyses (**Table 3.2a**).

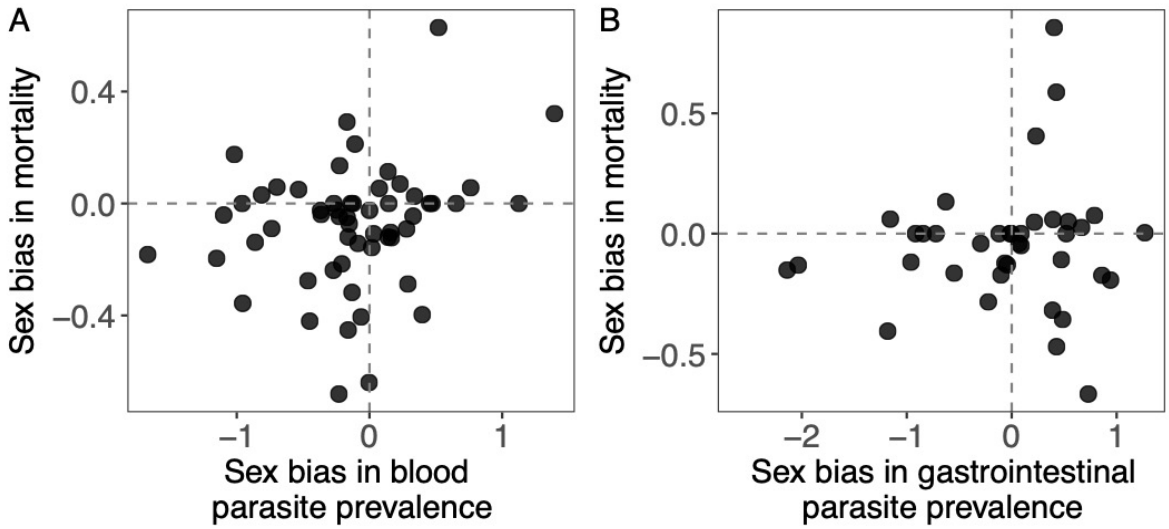

Fig. 3.2 Sex bias in annual mortality in relation to the bias in prevalence of (A) blood and (B) gastrointestinal parasites (see **Table 3.2** for statistics). Sex bias in mortality was expressed as log(male mortality/female mortality), whereas the sex bias in parasite prevalence was expressed as the weighted average effect size of all comparisons (see Material and methods). Dashed lines indicate no sex difference, positive values represent male bias and negative values female bias.

Discussion

To our knowledge, this work represents the largest comparative study of sex-specific parasite prevalence in birds, based on 96 species with sex-specific blood parasite prevalence data and 54 species with gastrointestinal parasite prevalence data. Taken together, our results showed little evidence supporting sex biases in parasite prevalence, with no overall sex bias in blood or gastrointestinal parasites prevalence in birds. Additionally, no relationship was found between sex bias in mortality and sex bias in parasite prevalence, even after controlling for possible confounding life history variables, i.e. mating system, body size and sexual size dimorphism.

Our findings disagreed with our prediction of male-biased parasitism generated by the sexual size dimorphism (Moore and Wilson 2002, Székely *et al*. 2007) and sex-different hormonal immunosuppression (Folstad and Karter 1992, Duffy *et al*. 2000). One possible explanation is that

in birds the magnitude of the difference in size between sexes tends to be smaller compared to mammals (Lindenfors *et al*. 2007), where this association between sexual size dimorphism and parasite prevalence has been shown (Moore and Wilson 2002). Furthermore, Ricklefs (1992) and McCurdy *et al*. (1998) found little and no relationship between body size and blood parasites across avian species. Scheuerlein and Ricklefs (2004) found an association in parasite prevalence and body size in passerines, however, after controlling for phylogeny the association was marginal. On the other hand, although stress and sex hormones were not part of our analysis, our results give little support the idea of sex-differences in corticosterone immunosuppression, and seem to be in line of with recent research finding inconclusive results in the immunocompetence handicap theory in birds (Roberts *et al*. 2004, Boyd *et al*. 2018, Kelly *et al*. 2018).

Specifically, we found no significant sex bias in the overall prevalence of blood parasites, consistent with the overall results of McCurdy *et al*. (1998). Sex differences in blood parasites are generally thought to occur due to unequal exposure of the sexes to vectors (van Oers *et al*. 2010, Egizi *et al*. 2014) and differences between males and females in the immune-endocrine system (Schuurs and Verheul 1990). Perhaps the lack of sex differences seen here could be attributed to these processes balancing each other out. For example, in males, the persistent pressure of male-male competition could generate stress-induced corticosterone which due to its immunodepressive effect could make them more prone to infection (Gao *et al*. 2017), at the same time that the elevated exposure of females to vectors while incubating (Korpimäki *et al*. 1993). Poulin (1996) found a strong male-biased infection of Acanthocephalan and Nematodes parasites, consistent with our results in the overall parasite prevalence in Nematoda. Nematodes are a very diverse group of round worms. Male-biased parasite prevalence in this group could be due to many non-exclusive variables including those previously suggested for overall gastrointestinal parasites (mainly based on differences in body size; see Introduction), in addition to sex-specific foraging behavior as result of niche specialization or competitive exclusion by the dominant sex (Mancini *et al*. 2013, Pereira *et al*. 2018). However, more studies are needed to test these hypotheses.

Mortality was not related to parasite prevalence across all analyses conducted, even in multi-predictor analyses where mortality was tested against parasite prevalence, body mass and mating system. Only body mass was consistently associated with mortality as found in previous studies(Liker and Székely 2005, Benito and González-Solis 2007, Székely *et al*. 2014a). Although parasite burden has often been linked to mortality in species-specific studies in birds (Møller and Nielsen 2007, Lachish *et al*. 2011, Adelman *et al*. 2017, but see Podmokła *et al*. 2017), here, we found that such association seems to be less clear at interspecific level. Nevertheless, our results should be treated cautiously because in most cases parasitism and mortality data did not come from the same population, and because our dataset consisted of studies that reported prevalence values for both sexes, which could represent a bias toward sexually dimorphic species. In addition, prevalence, as an index of parasitism, could be problematic because it informs about the proportion

of infected individuals in relation to the number examined (Margolis *et al*. 1982), generating uncertainty whether the individuals found positive only correspond to infection-resistant animals that survived the infection (van Oers *et al*. 2010). For example, Krementsov *et al*. (2017) found that males had lower survival than females after the viral infection, therefore, in the hypothetical situation of sampling this population in the wild without knowing this sex-different viral susceptibility beforehand, and assuming a similar infection rate between sexes, females would have a higher prevalence than males because a larger proportion of infected males died.

In contrast to the findings of Moore and Wilson (2002) in mammals, sex-biased parasitism in birds did not seem to be a consistent driver of sex-specific mortality. The pressure that parasites impose on birds not only appeared to be low between sexes but also within sexes as no increase nor diminution of mortality were seen when tested males and females separately. Perhaps, juveniles should be the target by further studies to obtain a thorough understanding of mortality patterns. Hints of this were shown by Eberhart-Phillips *et al*. (2017), Eberhart-Phillips *et al.* (2018), revealing that juvenile mortality rather than chick and adult mortality corresponded to the main contributor towards sex bias in ASR in six plover populations (*Charadrius*). Unfortunately, juvenile sex-specific parasitism data in birds is scant.

In conclusion, our analyses showed that birds did not exhibit overall sexual difference in parasite prevalence, and parasite prevalence did not predict sex-specific mortality, thus suggesting that other processes may drive the sex-differences in adult mortalities reported from numerous bird species. Though, perhaps the limitations of in our analysis (mentioned above) contributed to this lack of association. Although life history traits (e.g. mating system, parental care female reproductive investment and body mass) have been shown as important predictors of mortality in birds (Liker and Székely 2005, Benito and González-Solis 2007, Székely *et al*. 2014a), the actual etiology that originates female-biased mortality in birds is still poorly explored. Perhaps mortality events during migration (Hewson *et al*. 2016), predation (Xenophontos and Cresswell 2016), susceptibility to stress (Losdat *et al*. 2015), or simply resilience to starvation are more important determining sex-specific mortality than parasites. In addition to this, understanding male versus female immune systems undoubtedly is highly relevant. We call for further comparative and single-species studies to understand the causes of sex different mortality patterns.

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Table 3.1 Phylogenetic meta-analysis of sex difference in prevalence of blood parasites and gastrointestinal parasites. The estimate represents the weighted average effect size as Log odds ratio and its positive or negative value represents the sex bias directionality (see **Fig. 3.1**)

	Q_{REML} (*P*-value)	*k*	*n*	Studies	Estimate (95% CI)	*Z* statistic (*P*-value)
Prevalence of blood parasite (overall)	265.994 (0.043)	229	96	78	-0.024 (-0.130, 0.082)	-0.451 (0.652)
Haemoproteus	61.240 (0.575)	69	60	51	-0.117 (-0.444, 0.210)	-0.704 (0.481)
Leucocytozoon	39.710 (0.6559)	49	43	33	0.049 (-0.413, 0.511)	0.209 (0.835)
Plasmodium	30.820 (0.822)	44	39	29	0.022 (-0.220, 0.263)	0.178 (0.859)
Trypanosoma	17.257 (0.8375)	28	23	21	-0.080 (-0.443, 0.283)	0.186 (0.666)
Microfilaria	5.186 (0.878)	13	10	10	0.145 (-0.341, 0.632)	0.591 (0.555)
Prevalence of gastrointestinal parasites	226.818 (<0.001)	116	49	37	0.016 (-0.203, 0.234)	0.140 (0.889)
Cestoda	68.354 (<0.001)	27	23	22	-0.002 (-0.372, 0.368)	-0.011 (0.991)
Acanthocephala	6.141 (0.726)	12	10	10	0.137 (-0.444, 0.717)	0.461 (0.645)
Nematoda	37.544 (0.162)	33	22	20	0.388 (0.004, 0.773)	3.918 (0.048)
Trematoda	20.086 (0.389)	21	11	8	-0.252 (-0.885, 0.380)	-0.782 (0.434)
Protozoa	12.537 (0.484)	15	15	5	-0.596 (-1.708, 0.516)	-1.050 (0.294)

Note: Meta-analyses were performed using multivariate random-effect meta-analysis with restricted maximum likelihood (REML). Fixed-effect variables: period of sampling and method of parasite detection. Random-effect variables: phylogenetic relatedness and study. Q_{REML} = test for heterogeneity; *k* = number of effect sizes; *n* = number of host species; Studies = number of studies.

Table 3.2 Phylogenetic generalized least squares (PGLS) showing single-predictor and multi-predictor relationships between annual mortality and prevalence of (a) blood parasites and (b) gastrointestinal parasites. Multi-predictor models include two additional life history variables: body mass and mating system. First each sex was analyzed separately, then we tested the relationship between sex bias in the response and predictor variables (see Material and methods).

Response variable	Explanatory variable	Slope	*P*
(a)			
Single-predictor models (n = 56)			
Male annual mortality Adjusted R^2 = 0.00; λ = 0.647	Male overall blood parasite prevalence	0.069	0.378
Female annual mortality Adjusted R^2 = 0.00; λ = 0.584	Female overall blood parasite prevalence	0.068	0.354
Sex bias in annual mortality Adjusted R^2 = 0.03; λ = <0.001	Sex bias in blood parasite prevalence	0.089	0.098
Multi-predictor models			
Male annual mortality (n = 50)	Male overall blood parasite prevalence	0.056	0.233
Adjusted R^2 = 0.21; λ = 0.973	Male body mass	-0.043	0.005
	Male mating system	0.029	0.013
Female annual mortality (n = 49)	Female overall blood parasite prevalence	0.044	0.455
Adjusted R^2 = 0.27; λ = 0.706	Female body mass	-0.061	0.002
	Female mating system	0.064	0.031
Sex bias in annual mortality (n = 54)	Sex bias in blood parasite prevalence	0.071	0.136
Adjusted R^2 = 0.07; λ = <0.001	Sexual size dimorphism	0.015	0.908
	Sex bias in mating system	0.037	0.032

(b)			
Single-predictor models (n = 38)			
Male annual mortality Adjusted $R^2 = 0.00$; $\lambda = 0.829$	Male overall gastrointestinal parasite prevalence	-0.016	0.837
Female annual mortality Adjusted $R^2 = 0.00$; $\lambda = 0.934$	Female overall gastrointestinal parasite prevalence	0.019	0.779
Sex bias in annual mortality Adjusted $R^2 = 0.00$; $\lambda = 0.397$	Sex bias in gastrointestinal parasite prevalence	0.038	0.462
Multi-predictor models (n = 38)			
Male annual mortality	Male overall gastrointestinal parasite prevalence	0.030	0.652
Adjusted $R^2 = 0.39$; $\lambda = 0.721$	Male body mass	-0.092	<0.001
	Male mating system	0.023	0.217
Female annual mortality	Female overall gastrointestinal parasite prevalence	-0.006	0.931
Adjusted $R^2 = 0.28$; $\lambda = 0.806$	Female body mass	-0.089	0.001
	Female mating system	0.030	0.298
Sex bias in annual mortality	Sex bias in gastrointestinal parasite prevalence	0.031	0.493
Adjusted $R^2 = 0.19$; $\lambda = 0.603$	Sexual size dimorphism	-0.736	0.004
	Sex bias in social mating system	-0.015	0.637

Supplementary material

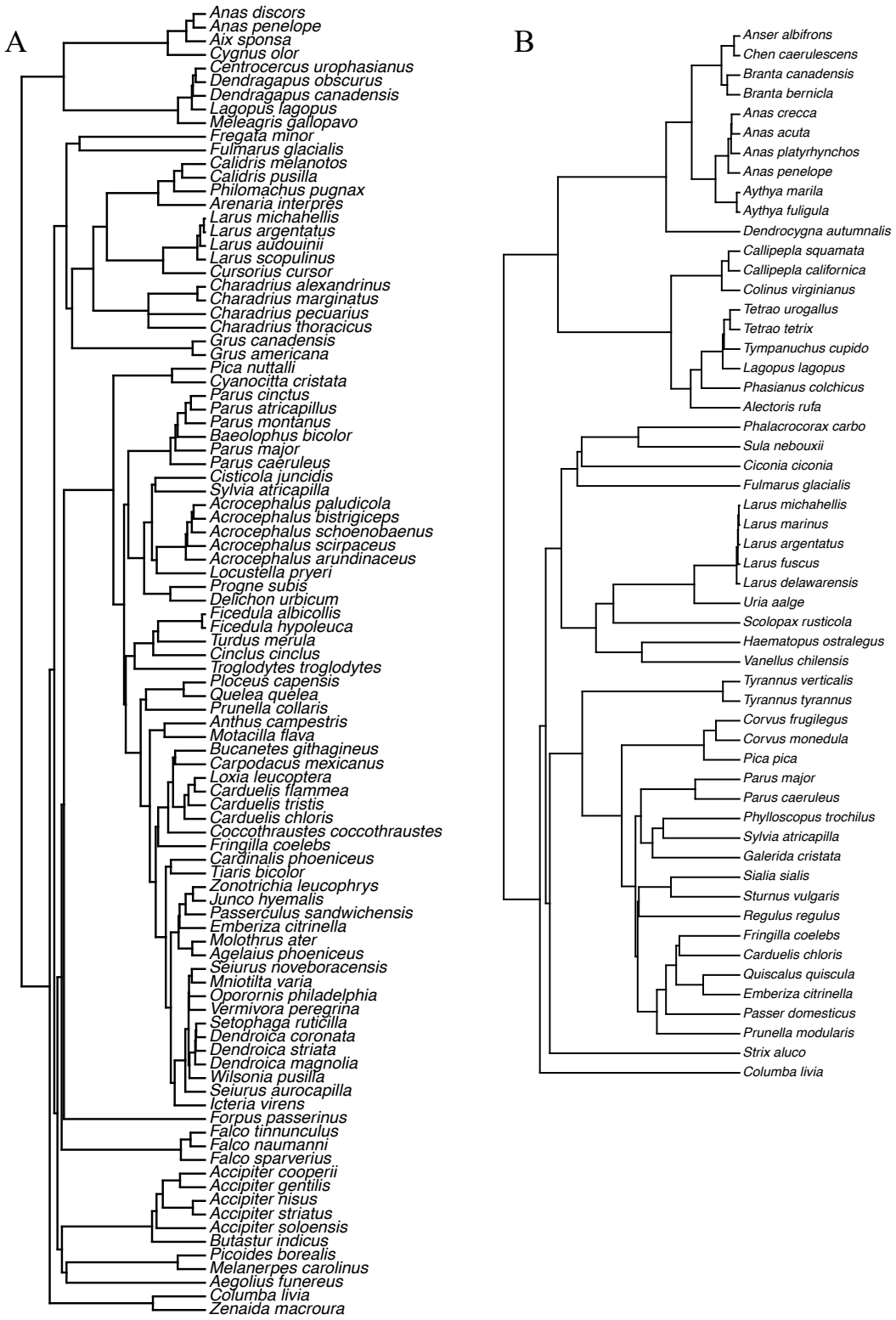

Figure S3.1 Phylogenetic hypothesis used in the comparative analysis for (A) blood parasites and (B) gastrointestinal parasites

Appendix V | Seasonal variation in sex-specific immunity in wild birds

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Author's Contributions

J.O.V. collected the data, conducted the data analysis and wrote the paper.

All authors contributed substantially to study design and revisions of the paper.

Abstract

Males and females differ in many traits that could lead to sex differences in survival. Survival is aided by the immune system, a major physiological axis that defends hosts against pathogens. Whilst the immune system often varies seasonally and exhibit differences between males and females, the general patterns in seasonality and sex differences across taxa have remained controversial. Birds are excellent model organisms to assess these patterns, because the immune system of many species is well characterised. We conducted a meta-analysis using 41 wild bird species from 24 avian families to investigate sex differences and seasonal (breeding/non-breeding) variations in immune status, including white blood cell counts, phytohaemagglutinin (PHA) test, bacteria-killing ability (BKA), haemolysis and haemagglutination assays. We found male-biased macrophage concentration, BKA and haemolysis titers, but only during the breeding season. Sex-specific heterophil concentrations, heterophil/lymphocyte ratios and PHA responses differed between breeding and non-breeding, suggesting larger changes in males than in females. Importantly, sex differences in immune status is stronger during the breeding period than during the non-breeding period. Taken together, our study suggests that both seasonal variation and sex differences in immune system are common in birds, although their associations are more complex than previously thought.

Keywords

seasonality, sexual dimorphism, sex difference, breeding season, immunocompetence, mortality

Introduction

Sex-specific mortality has been reported across many animal taxa (Moore and Wilson 2002, Wiklund *et al*. 2003, Székely *et al*. 2014). However, the proximate causes of why one sex outlives the other are often unclear or ignored. Sex differences in life history, behaviour, and physiology are nearly ubiquitous in nature, with numerous traits showing distinctive patterns for males and females that could relate to differences in survival. Examples include sex differences in spatial segregation of foraging areas (Pereira *et al*. 2018), natal and breeding dispersal (Mabry *et al*. 2013, Végvári *et al*. 2018), parasite prevalence, or survival from viral infection (Korpimäki *et al*. 1993, Moore and Wilson 2002, Krementsov *et al*. 2017). Although these variables may contribute to sex differences in mortality, the role of one fundamental physiological axis that is expected to make major contributions to survival still remains relatively unexplored: the immune system.

The immune system is complex and involves both cellular and humoral responses that help the host to thwart off attacks from pathogens (Abbas *et al*. 2015). Differences in immune response between the sexes have been described extensively across vertebrates. These sex differences have been traditionally associated with the immunomodulating effect of sex hormones, where oestrogens, found in higher concentrations in females, act as weak immune-enhancers, and androgens, higher in males, as immune-suppressors (Furman *et al*. 2014, Klein and Flanagan 2016). However, these studies have been centred primarily on humans and laboratory animals, while there is increasing evidence suggesting that the association between sex hormones and sex differences in immunity in the wild are not as simple as first thought. Two independent meta-analysis showed that testosterone did not have a consistent overall immunosuppressive effect in males, and the effect depended on the taxa studied and whether the experimental manipulations involved hormone concentrations above physiological levels (Roberts *et al*. 2004, Foo *et al*. 2017). A recent study has also challenged the notion of sex biases in immunity by finding no overall sex difference in immune estimates in a large-scale comparative analysis including vertebrates and invertebrates (Kelly *et al*. 2018). However, Kelly *et al*. (2018) showed that some patterns do arise when focusing on specific immune variables and taxonomic groups, such as mammals, which showed a strong male bias in specific pro-inflammatory cytokines. For birds, Kelly *et al*. (2018) did not find overall sexual differences in immunity, but they concluded that future studies of sex differences in immunity should include variables known to affect immune functioning, such as age (Tella *et al*. 2002), nutritional state (Korver 2012), photoperiod (Demina *et al*. 2019) or seasonality (Martin II *et al*. 2004). The latter variable is especially relevant, because seasonal changes, in particular the transition between the non-breeding and the breeding period, involve major physiological and behavioural changes. They may also include pronounced environmental shifts, particularly in species that migrate between breeding and non-breeding grounds, which is the case in many species of birds.

Accordingly, several studies have found important sex-specific changes in immunity between the non-breeding and breeding period in birds. For example, Hõrak *et al*. (1998) found that female Great Tits, *Parus major*, had more circulating lymphocytes than males in spring but not in summer. Merrill *et al*. (2013) found that male Brown-headed Cowbirds, *Molothrus ater*, showed higher bactericidal capacity than females during the breeding period compared to the non-breeding period. Reasons behind such complex seasonal, species-specific and sex-specific immunity are not fully understood. Recurring explanations include sex-specific energetic and nutritional costs that may be traded off against immunity (Nilsson *et al*. 2007, Marais *et al*. 2011, but see Hasselquist and Nilsson 2012), thus resulting in an impaired immunity in the sex with higher energy expenditure (e.g. courtship displays, egg production, parental care, Bryant and Westerterp 1980, Maxson and Oring 1980, Brunton 1988).

Alternatively, immune defence may be compromised in situations that cause strain or tension, i.e. stress (Merrill *et al*. 2012). Corticosterone, the main circulating glucocorticoid in birds, could play an important role here. First, because corticosterone is involved in regulating the metabolism (Romero 2002), and second, as result of increase in stress-induced corticosterone production (e.g. during territory defence) that could supress immune function (Matson *et al*. 2006, Cyr *et al*. 2007, Gao *et al*. 2017). However, a comprehensive analysis that simultaneously investigates seasonally-related and sex-biased immunity across bird species is largely lacking. Also, it is unknown whether potential sex-specific or seasonal patterns are consistent between immune parameters (Palacios *et al*. 2018).

Here, in order to better understand the variation in avian immune function, we conducted a meta-analysis to test for seasonal (breeding *versus* non-breeding season) and sexual differences in immunity across bird species. Because of the known effects of ontogeny and captivity on immunity (e.g. Martin *et al*. 2011, Jakubas *et al*. 2015), we restricted our analysis to data from free-living adult birds. We included information from nine measurements characterizing immune status: the relative frequency of four types of white blood cells (heterophils, lymphocytes, macrophages, eosinophils), the ratio of heterophils/lymphocytes (H/L ratio, a glucocorticoid-mediated immune index of stress), and four widely used immune response indexes (the phytohaemagglutinin test, bacteria-killing ability assay, haemolysis assay, and the haemagglutination assay). For each of these nine immune parameters we estimated their overall meta-analytic means (i.e. estimates of sex-specific immune biases). Based on previous studies (Lee 2006, Kelly *et al*. 2018), we expected no sex difference in white blood cells levels and a small female bias in the immune response indexes. Next, we broke down these overall estimates by season, and computed one estimate for the non-breeding period and one for the breeding period. This allowed us to test if these seasonal estimates were sex-biased, and if season, as a variable, had a significant effect on the immune parameters. Because breeding often incurs increased workload, higher energy demands, and stressful situations compared to non-breeding

birds in winter (Hasselquist and Nilsson 2012), we expected the two periods to differ from each other, and season to significantly affect immune variables (e.g. Nordling *et al*. 1998, Merrill *et al*. 2015).

Furthermore, we used the estimates from male and female individuals to test if the sexes could respond differently to the transition between seasons. Males are generally more involved in courting behaviour and intrasexual aggression; therefore, we predicted a possible stress-mediated immunosuppression (Gao *et al*. 2017) in males that could outweigh an alternative immunosuppression due to energetic trade-offs in females (Brunton 1988). Thus, in the transition from non-breeding to breeding, males may exhibit stronger changes in immune estimates than females.

Materials and methods

Literature search

We systematically collected sex-specific white blood cells and immune response data from birds (PRISMA method, Moher *et al*. 2009) using ISI Web of Science (see chart in **Fig. S6.1**; list of references in supplementary material). Our inclusion criteria required these data to be: (i) determined from adult birds with known sex, (ii) obtained from free-living wild birds (not captive), and (iii) from populations that were not experimentally manipulated. In order to conduct the meta-analytic calculations, the selected studies should provide the number of individuals examined per sex, the arithmetic mean of the immune variable measured and an estimate of its variance. We only included publications reporting results for both sexes to avoid difficulties generated by different sampling/diagnostic methods or different populations when calculating individual effect sizes.

Immune variables

White blood cells (WBC). We used data on the four most abundant WBC circulating in avian blood (Davison *et al*. 2008): heterophils, lymphocytes, macrophages (also known as monocytes), and eosinophils. Basophils counts were discarded because of insufficient data available. The H/L ratio was also collected or calculated using the raw values of heterophils and lymphocytes. Elevated leucocyte number is a symptom of a stress syndrome, inflammatory processes and/or oxidative stress (Davis *et al*. 2008). Usually, leucocytosis is caused by an elevated concentration of heterophils and/or lymphocytes (Dein 1986, Ots *et al*. 1998). Lymphocytes are immune cells that assist in the recognition and destruction of many types of pathogens. Although sometimes difficult to interpret, decreased lymphocyte concentrations may signal stress-induced immunosuppression (Hõrak *et al*. 1999), or may indicate a lack of parasite infections (Ots and Hõrak 1998). Heterophils are non-specific phagocytic cells that enter the tissues during inflammatory processes. Heterophil concentrations increase as response to inflammatory processes, stress and infections (Ots *et al*. 1998). Thus, the ratio of these two cell lines is considered a reliable proxy of physiological stress

in birds (Davis *et al*. 2008, Skwarska 2019). Macrophages and eosinophils are less prominent in the avian blood and their main function is to phagocyte and present antigens to lymphocyte T (T-cells), and mediate the defence against parasite infections, respectively, and variation in their levels is commonly associated with pathogen infection (Davison *et al*. 2008). WBC data came from apparently healthy individuals, therefore assumed to represent baseline levels. The time between capture and sampling was not always available (details in **Table S6.1**), and Davis (2005) showed that within one hour of capture the total leucocyte counts decreased as a result of handling stress, whereas proportions of each leucocyte type did not differ significantly. Therefore, we calculated WBC proportions (from the total number of leucocytes) to reduce between-study variation.

Estimates of immune response. We used four widely accepted measures of immune response in birds: the (i) phytohaemagglutinin test (PHA), that consists of a subcutaneous injection of this mitogen (phytohaemagglutinin) that triggers a local immune response mediated mostly by T-cell infiltration. Components of innate and adaptive immune system take part in the response and is estimated by measuring the degree of swelling of the skin, usually 24 h post-injection (Martin II *et al*. 2006). The (ii) bacteria-killing ability assay (BKA) quantifies the ability of proteins in the plasma (such as complement, natural antibodies, and lysozymes) and/or phagocytic cells to kill bacteria (French and Neuman-Lee 2012). The (iii) haemolysis and (iv) haemagglutination assays use foreign red blood cells (usually rabbit) to quantify titres of complement-like lytic enzymes (i.e. lysis, HL) and non-specific natural antibodies (i.e. agglutination, HA) in plasma (Matson *et al*. 2005). From each study we recorded whether the study was done during the breeding or the non-breeding season (hereafter season). Details of the breeding status extracted from each study are presented in **Tables S6.1** and **S6.2**.

We used standard deviation (SD) as estimate of variance. When standard error was provided, we calculated SD using equation (1):

$$SD = SE \times \sqrt{n}, \quad (1)$$

where SE is the standard error, and n is the sample size.

When 95% confidence intervals were given (in two studies), SD was calculated with equation (2):

$$SD = \frac{\sqrt{n} \times (upper\ CI - lower\ CI)}{2 \times \delta}, \quad (2)$$

where n is the sample size, CI the confident intervals, and δ is the value for the *t*-distribution with degrees of freedom equal to the sample size minus 1 and a probability of 0.05 (for details, see Higgins and Deeks 2008).

Statistical analysis

Phylogenetic meta-analysis. To investigate sex biases in immunity, a phylogenetic multivariate meta-analysis was performed using the R package *metafor* (Viechtbauer 2010). Effect sizes were computed using Hedge's *g* for standardised means because of its common use in ecology literature and for including a correction for small sample sizes (Hedges 1981, Rosenberg *et al*. 2013). Effect sizes are the standardised mean difference between two groups, which in our case corresponded to the mean of males relative to the female mean. Negative values of *g* indicate female bias in the immune parameter studied and positive values a male bias. We conducted multivariate random-effect meta-analyses using the previously computed effect sizes as response variable and season (non-breeding/breeding) as moderator (i.e. fixed-effect). Phylogeny (a variance-covariance matrix) and study (to account for more than one species and/or immune estimate per study) were added as random-effect variables. We used the avian phylogeny proposed by Jetz *et al.* (2012) and the analyses were conducted using consensus trees (one for each type of immune variable, **Fig. S6.2**) obtained by 50% majority-rule (Holder *et al*. 2008, Sukumaran and Holder 2010) from 1,000 randomly selected trees from a pool of 10,000 available trees (http://birdtree.org) using the methodology described by Rubolini *et al*. (2015). These phylogenetic trees were not fully resolved, and polytomies were arbitrarily resolved by adding a branch distance of 10^{-8} to one randomly chosen branch in the polytomy using the function "multi2di" from the R package *ape* (Paradis *et al*. 2004). Publication bias (due to missing studies that were not published because of negative or null results, Koricheva *et al*. 2013) was evaluated by inspecting the symmetry in funnel plots and using the Egger's regression test (Egger *et al*. 1997, Sterne and Egger 2005) by including the standard error of the effect sizes as an additional moderator within the model. If the intercept significantly deviated from zero (significance of $P < 0.10$, Egger *et al*. 1997), the overall relationship between the precision and size of studies included in the data set was considered asymmetrical or, in other words, biased (Sterne and Egger 2005). Of the nine fitted models, only macrophages and eosinophils suggested presence of publication bias (both $P < 0.001$). Diagnostic tests for identifying influential data points and outliers, and rules for excluding these types of cases are not well established, particularly for multivariate/multi-level meta-analytical models (Viechtbauer and Cheung 2010). We used the approach described by Habeck and Schultz (2015), consisting of identifying the influential outliers causing the bias and running the models after excluding these values. We report results after removing one effect size from the final model of macrophages, and two from the model of eosinophils (see **Table S6.3** for the final sample sizes used in the analyses). The effect of season on the immune sex-bias was tested using the Omnibus test (*QM*) for moderators (a Wald-type Chi-squared) implemented within the function "rma.mv" (*metafor* R package), which tests whether the explained heterogeneity by a parameter (here, season) is significantly greater than the unexplained overall heterogeneity (Viechtbauer 2010). The HL and HA assays were excluded from further analysis, because only estimates of breeding birds were available. We used Cochran's *Q* test to estimate whether the (residual) heterogeneity among effect

sizes was greater than expected by sampling error alone (Borenstein *et al*. 2009). We also calculated the variance in effect sizes due to phylogenetic relatedness ($I^2_{\text{phylogeny}}$), differences among studies (I^2_{study}), and the total variance attributed to the random effect variables (i.e. the addition of the two effects, I^2_{total}).

Generalised linear mixed models. To explore if seasonal changes affected the sexes independently, we fitted generalised linear mixed models by Markov chain Monte Carlo techniques using the R package *MCMCglmm* (Hadfield 2010). This analysis differs from the previous in that here we analysed variation of each sex parameters according to season, instead of one 'combined' effect size. This approach helps to understand how each sex responds to season, because changes in effect size estimates from the non-breeding period to the breeding period may be the result of increases or reduction in one or both sexes at once. Each of these seven models (HA and HL were excluded) had immune variables as response variable, and season, sex (females/males), and the two-way interaction of season and sex as explanatory (fixed-effect) variables. All models included study and phylogeny as random-effect variables. The H/L ratio was log-transformed. The H/L ratio and PHA models were run with a Gaussian family distribution. The rest of the models were run using a binomial family distribution. To investigate whether the above comparisons may have been confounded by different species composition in the breeding and non-breeding samples, we ran these models two times. First using the full dataset, and then using a subset of the data that included only those species for which we had data from both non-breeding and breeding seasons (**Table S6.4**). We used parameter expanded (random-effects) and inverse-Wishart priors (fixed-effects) based on improving model convergence. Further details of model specification are given in the supplementary material. Convergence and autocorrelation levels were assessed through the Gelman-Rubin test (Gelman and Rubin 1992), trace graphs and the "autocorr" function, implemented in the R package *coda* (Plummer *et al*. 2006). MCMCglmm results are expressed as posterior mean, lower and upper 95% credible intervals, and significance as a pMCMC value.

Results

Sex biases in immunity and the effect of season (meta-analysis). Our results show that across all immune variables, while there was no overall difference between males and females (**Fig. 6.1A**), there was an important variation in sex differences between the non-breeding and the breeding period (**Fig. 6.1B**; **Table 6.1**). Macrophage concentration, haemolysis score and PHA response were significantly male-biased during breeding (**Fig. 6.1B**). During the non-breeding period, BKA tended to be higher in males (P = 0.089) while heterophil concentration tended to be higher in females (P = 0.079). Both phylogeny and study explained an important proportion of the variance in immune variables (**Table 6.1**).

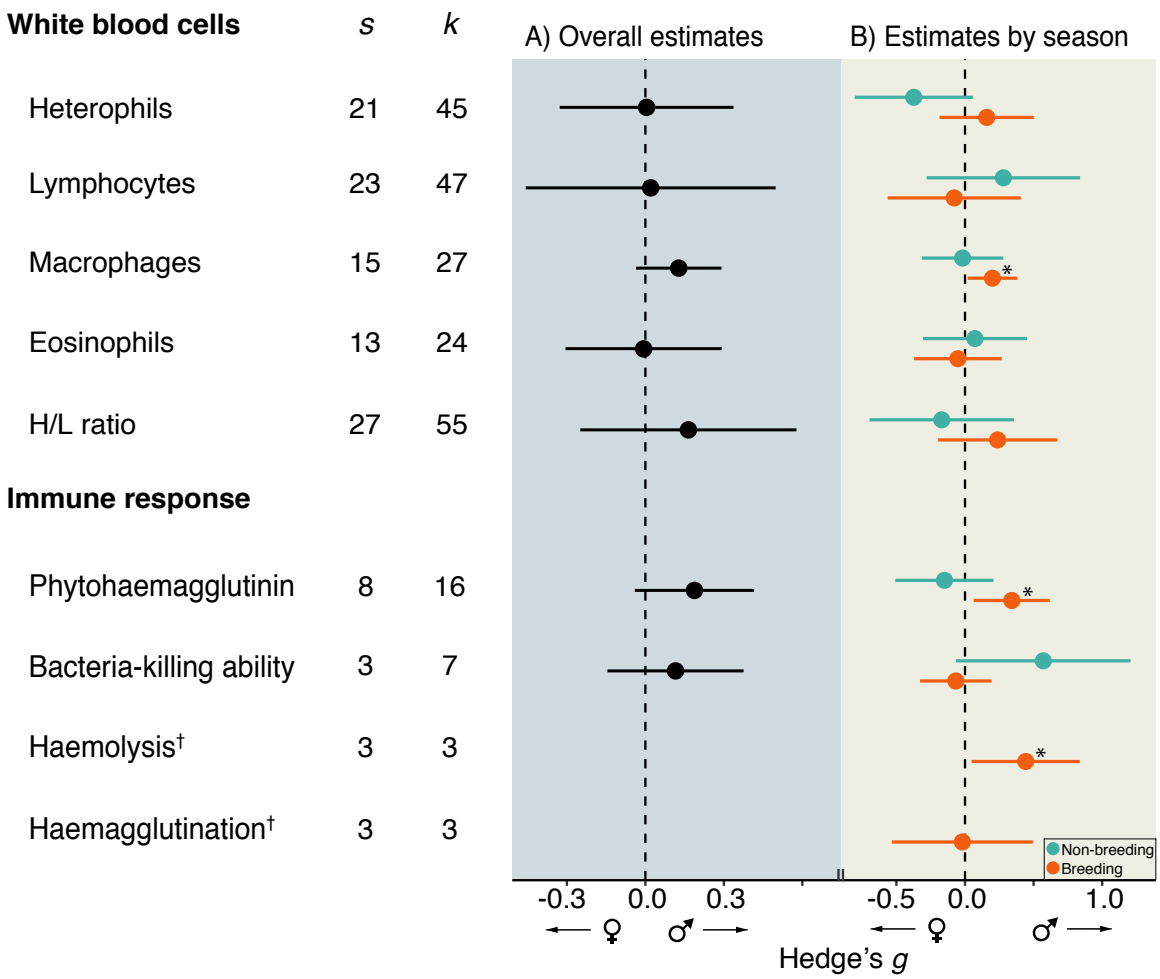

Figure 6.1 Sex bias in white blood cells and immune response assays in adult wild birds (weighted average effect sizes and 95% confidence intervals). A) Overall in immune estimates. B) Immune estimates for non-breeding (in cyan) and breeding (in orange) birds. Weighted averages were tested whether they differed significantly from zero (i.e. no sex bias, dashed line; see statistics in **Table 6.1**). *s* = number of species; *k* = number of effect sizes; H/L ratio = heterophils/lymphocytes ratio; *statistical significance ($P < 0.05$); †data from breeding birds only.

Seasonal changes had a significant effect on the sex bias estimates of three immune parameters: heterophil concentration, H/L ratio and PHA response (Omnibus test of coefficients [df = 1]: 8.131, $P = 0.004$; 8.547, $P = 0.003$; 4.832, $P = 0.028$, respectively; **Table 6.2**). These results indicate that, in these immune parameters, the immune estimates from the non-breeding and breeding periods were significantly different from each other. In all cases the direction of the skew was towards males. A non-significant trend in the opposite direction was found for lymphocyte concentration and for BKA, where estimates obtained in the breeding season deviated towards females (**Table 6.2**).

Effect of seasonal changes on males and females (GLMM analysis). The GLMM-MCMC models revealed a significant interaction between season and sex for heterophil concentration and H/L ratio, indicating that these variables show a greater change between non-breeding and breeding season in males than in females (**Fig. 6.2A**, **E**; **Table 6.3**). These results were consistent between models using the whole data set and those using a subset of species for which data during both the non-breeding and breeding season were available (**Table S6.5**). Also, for BKA, seasonal changes tended to differ between males and females when tested with the full data set ($P = 0.078$), but the pattern became weaker when using the subset of data (**Table S6.5**), arguably due to low sample size in this variable (**Fig. 6.2G**). The other immune parameters (lymphocytes, macrophages, eosinophils, PHA) showed no significant sex differences in the change between non-breeding and the breeding period, suggesting that males and females either increase or decrease their levels in comparable proportions (**Fig. 6.2**, **Table 6.3**).

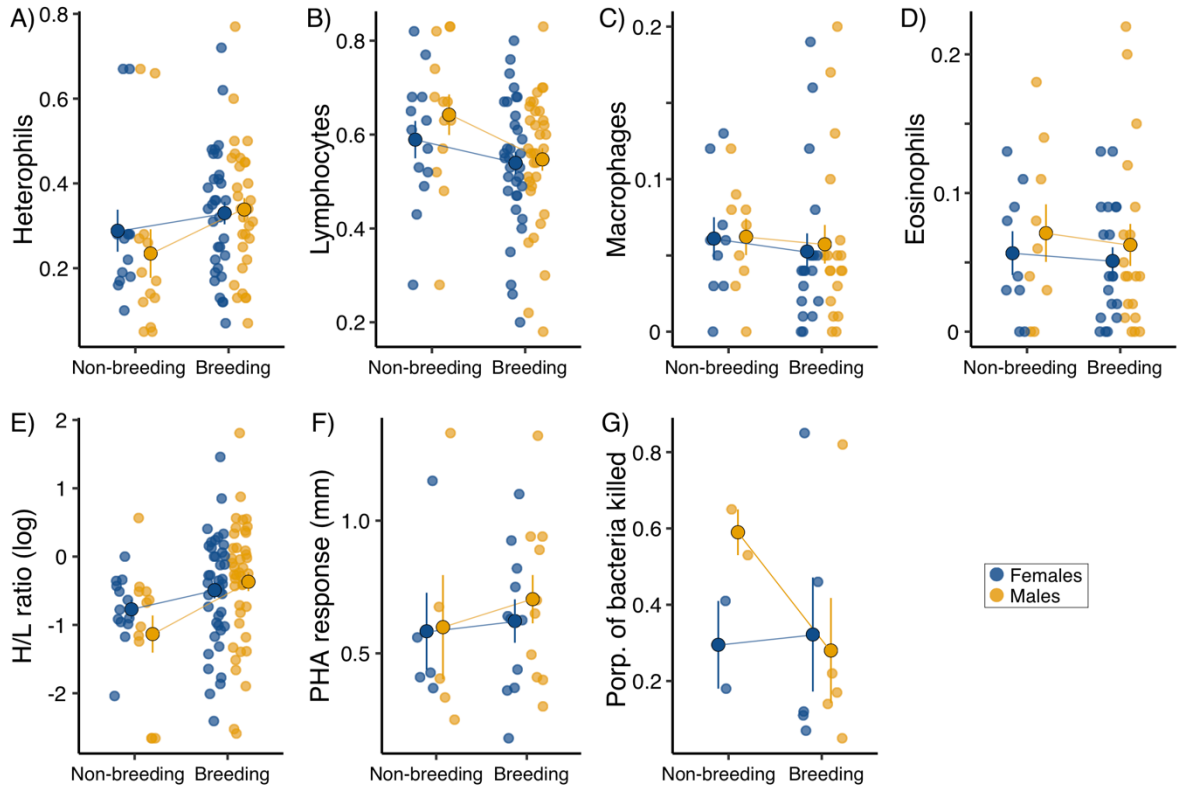

Figure 6.2 Characteristics of the immune system in wild birds. White blood cells (A-D, expressed as the proportion of the total white blood cell count), heterophils/lymphocytes (H/L) ratio (E), phytohaemagglutinin response (F), and bacteria-killing ability (G) in breeding and non-breeding birds (blue and yellow dots refer to females and males, respectively). Large outlined dots and whiskers are arithmetic means and standard errors, respectively.

Discussion

To our knowledge, this is the first multi-species analysis investigating the effect of seasonal variation on sex-specific immunity in wild birds. We showed an overall lack of sex differences in

the immune variables studied. However, when taking season into account, subtle but consistent patterns arise indicating that males are undergoing more substantial reorganization of their leukocyte composition during reproduction than females.

Similar to Kelly *et al.* (2018), the overall meta-analysis of the immune parameters showed no significant sex biases in immunity, although with subtle variations of male and female biases in the estimates. In multivariate meta-analysis, non-significant results could originate from small effect sizes being close to zero (i.e. no sex difference). However, the heterogeneity attributed to random effect variables was rather high (Q test and I^2) (Higgins *et al*. 2003), suggesting that our data set had great variation of opposing effect sizes (i.e. some species estimates showing a male bias and others a female bias). Breaking the immune estimates down by season revealed notable sex differences between the non-breeding and breeding period, with macrophage concentration, PHA response and haemolysis score being male biased, and a significant seasonal influence on the estimated sex bias for heterophil concentration, H/L ratio and PHA response. Heterophils and lymphocytes make up to 95% of the total leucocyte count (Clark *et al*. 2009). Both cell types have important roles in innate immunity, but only lymphocytes participate in adaptive immunity (Dein 1986, Davison *et al*. 2008). Macrophage levels were male-biased during the breeding period, but no sex differences were found for levels of eosinophils. Macrophages and eosinophils are specialised against unspecific cells like apoptotic cells or microbes, and against parasite infections, respectively (Abbas *et al*. 2015). Studies reporting sex differences in these two leucocyte lines in birds are scarce. Variation in levels of eosinophils are attributed to different levels of infection by gastrointestinal parasites in birds (Dehnhard and Hennicke 2013, Gallo *et al*. 2019), and sex differences in macrophage gene expression associated to the sex chromosomes have been reported in chicken (Garcia-Morales *et al*. 2015).

Seasonally varying levels in stress (defined as a physiological response due to strain or tension), hormones and workload may form the basis of mechanisms that could explain our findings. First, stressors associated with breeding could cause immunosuppression due to elevated glucocorticoid production (Gao *et al*. 2017, Gao and Deviche 2019). In most species, males are holding and defending the territory, being more involved in stressful activities than females during breeding (Prum 2017), which could lead to higher corticosterone production in males (Bókony *et al*. 2009, but see Clark *et al*. 2018, Davies *et al*. 2018). High H/L ratios may reflect immunosuppression by stress in birds (Davis *et al*. 2008, Skwarska 2019), which could explain our results in this variable. Although the H/L ratio was not sex-biased either during the non-breeding or the breeding season, both estimates were different from each other, and males experienced a greater increase than females between the seasons. Second, the breeding period in birds is characterised by behavioural changes triggered by the sex hormones. Androgens and oestrogens have traditionally been thought to influence immunity in birds by up- or down-regulating their immune system. However, current evidence disregards sex hormones (mostly testosterone) as

important immune modulators in birds (Roberts *et al*. 2004, Foo *et al*. 2017). For instance, Roberts *et al*. (2009) found no effect of testosterone on immune response in Japanese Quail, *Coturnix japonica*. Li *et al*. (2015) found that in Eurasian Tree Sparrow, *Passer montanus*, testosterone concentration was positively correlated with the strength of PHA response in males, whereas in females the correlation was negative. Additionally, Duffy *et al.* (2000) concluded that the increase in plasma corticosterone upon treatment with testosterone implants in European Starlings, *Sturnus vulgaris*, was the likely cause of immunosuppression in males and females rather than testosterone itself. However, conclusions from studies in wild birds have been based mainly on correlational observations, which may obscure the real effect of sex hormones on immunity.

Third, specific stages of reproduction require temporarily elevated energy and nutrient input, which could compromise immune function (Bourgeon *et al*. 2010, Hasselquist and Nilsson 2012, Cabrera-Martínez *et al*. 2018). However, we deem such potential mechanism of little relevance in explaining our findings, because estimates of immune function in our data were obtained from species with different breeding systems and, moreover, at various moments throughout the entire breeding period. Unfortunately, with the available data we were unable to divide the breeding period further into stages (e.g. courting, incubation, brood care) to determine if changes in immune status are associated to one or several of these breeding stages.

Although data on immune response variables were not available for many species, we did find differences between males and females. The four immune assays analysed reflect innate immunity, except the PHA test that, if repeated more than once, also includes components of the adaptive immunity (Santiago-Quesada *et al*. 2015). The PHA test and the haemolysis assay were significantly male-biased during breeding, although the latter estimate was obtained from only three effect sizes. Generally, the PHA response in birds appears to decrease with the stress of breeding (Moreno *et al*. 1998, Martin II *et al*. 2006), although no association with breeding was found in Chinstrap penguins, *Pygoscelis antarctica* (Palacios *et al*. 2018). In Eurasian Tree Sparrow, Li *et al*. (2015) found no differences in PHA responses between breeding males and females, while Zhao *et al*. (2017) found that body condition but not breeding stage correlated with their haemolysis levels. Interestingly, in our analysis the PHA test and the BKA assay showed opposite responses to season (**Fig. 6.1B** and **Fig. 6.2F–G**). In both cases the differences seemed to be largely driven by changes in males (**Fig. 6.2F–G**). However, with a relatively small sample size and considering the subset analysis, the results of the model interaction of BKA assay should be taken cautiously. Alternatively, an explanation based on the sexual selection theory predicts higher innate immune response in the competing sex (usually males). According to this postulate, because the competing sex is more involved in aggressive interactions causing physical injury, selection would favour strong inflammation responses as an aid for healing wounds (Zuk and Johnsen 1998, Hasselquist 2007). The inclusion of mating system should thus be considered in future studies in order to test this hypothesis.

Here we have shown that across wild birds, sex differences in immunity associated to the breeding season may occur. The causes of these seasonal patterns of sexual changes in immune function are difficult to identify. In addition to the complex nature of the avian immune system, a number of unaccounted variables could directly or indirectly confound our analysis, such as genetic, environmental and ecological factors (like photoperiod or mate competition), with the potential of affecting one or several immune components, and in different sex-specific fashion. The scarcity of available studies to date prevented us also from exploring factors like mating system and parental care, which seem important to further understand the causes of seasonal and sexual changes in immunity. Nonetheless, our results highlight sexual differences in immune status as a relevant topic that requires further attention in wild birds.

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Competing interests

The authors declare no competing interests.

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Table 6.1 Sex bias in (a) white blood cell types and the H/L ratio, and (b) immune response assays in adult wild birds. *P*-values < 0.05 in bold.

Immune variable	I^2 (%) phylogeny	I^2 (%) study	I^2 (%) total	Q_{REML} (*P*)	Overall estimates		Estimates by season			
					Overall (95% CI)	*Z* statistic (*P*)	Non-breeding (95% CI)	*Z* statistic (*P*)	Breeding (95% CI)	*Z* statistic (*P*)
a) White blood cells										
Heterophils	23.69	46.08	69.76	171.238 (<0.001)	0.005 (-0.327, 0.337)	0.027 (0.978)	-0.373 (-0.804, 0.057)	-1.698 (0.089)	0.158 (-0.186, 0.502)	0.902 (0.367)
Lymphocytes	45.17	33.91	79.07	182.957 (<0.001)	0.020 (-0.457, 0.498)	0.084 (0.933)	0.280 (-0.279, 0.839)	0.981 (0.327)	-0.079 (-0.564, 0.406)	-0.318 (0.750)
Macrophages	<0.01	22.98	22.98	26.780 (0.367)	0.128 (-0.036, 0.291)	1.531 (0.126)	-0.018 (-0.314, 0.279)	-0.117 (0.907)	0.200 (0.020, 0.380)	**2.175 (0.030)**
Eosinophils	38.52	0.00	38.52	26.520 (0.277)	-0.007 (-0.305, 0.292)	-0.045 (0.964)	0.073 (-0.307, 0.452)	0.375 (0.708)	-0.052 (-0.371, 0.268)	-0.317 (0.752)
H/L ratio	40.18	33.39	73.58	191.669 (<0.001)	0.143 (-0.296, 0.582)	0.639 (0.523)	-0.171 (-0.700, 0.358)	-0.634 (0.526)	0.240 (-0.199, 0.680)	1.071 (0.284)
b) Immune response										
PHA	0.00	9.54	9.54	13.839 (0.462)	0.188 (-0.040, 0.415)	1.614 (0.107)	-0.150 (-0.508, 0.208)	-0.821 (0.412)	0.341 (0.063, 0.619)	**2.407 (0.016)**
BKA	<0.01	0.00	<0.01	15.122 (0.010)	0.115 (-0.145, 0.376)	0.868 (0.385)	0.571 (-0.066, 1.207)	1.758 (0.079)	-0.067 (-0.328, 0.194)	-0.503 (0.615)
HL	33.39	0.00	33.39	2.080 (0.354)	–	–	–	–	0.443 (0.048, 0.837)	**2.199 (0.028)**
HA	28.45	33.48	61.93	4.605 (0.100)	–	–	–	–	-0.019 (-0.533, 0.495)	-0.074 (0.941)

H/L ratio = heterophils/lymphocytes ratio, PHA = phytohaemagglutinin test, BKA = bacteria-killing ability assay, HL = haemolysis assay, HA = haemagglutination assay. $I^2_{phylogeny}$ = variance due to phylogenetic relatedness, I^2_{study} = variance due to differences among studies, I^2_{total} = total variance attributed to the random effect. Q_{REML} = Cochran's *Q* test for (residual) heterogeneity. *Z* statistic tests if immune parameter estimate differ from zero (no sex difference).

Table 6.2 Omnibus test of coefficients (*QM*) testing for the effect of season on the sex bias of the immune parameters studied. *P*-values < 0.05 in bold.

Immune variable	*QM* (df = 1)	*P*
White blood cells		
Heterophils	8.131	**0.004**
Lymphocytes	3.453	0.063
Macrophages	1.662	0.197
Eosinophils	0.488	0.485
H/L ratio	8.547	**0.018**
Immune response		
PHA	4.832	**0.028**
BKA	3.301	0.069

H/L ratio = heterophils/lymphocytes ratio, PHA = phytohaemagglutinin test, BKA = bacteria-killing ability assay.

Table 6.3 White blood cell levels and immune responses in wild birds in relation to sex and season (MCMC generalised linear mixed models; *n* = total number of individuals; *s* = number of species). *P*-values < 0.05 in bold.

		95% credibility intervals		
	Post. mean	Lower	Upper	*P*
a) Heterophils (*n* = 90, *s* = 21)				
Intercept	-0.448	-1.336	0.338	0.236
Season (breeding)[a]	-0.113	-0.579	0.317	0.602
Sex (males)[b]	-0.396	-0.723	-0.124	**0.012**
Season (breeding)[a]*sex (males)[b]	0.429	0.036	0.760	**0.018**
Random				
Study	0.560	0.070	1.069	
Phylogeny	0.411	< 0.001	1.310	
Residual	0.081	0.033	0.136	
b) Lymphocytes (*n* = 94, *s* = 23)				
Intercept	-0.091	-0.768	0.548	0.782
Season (breeding)[a]	0.035	-0.405	0.393	0.864
Sex (males)[b]	0.254	-0.026	0.580	0.094
Season (breeding)[a]*sex (males)[b]	-0.221	-0.570	0.148	0.238
Random				
Study	0.170	< 0.001	0.426	
Phylogeny	0.336	< 0.001	0.840	
Residual	0.109	0.052	0.163	
c) Macrophages (*n* = 56, *s* = 15)				
Intercept	-3.494	-5.220	-1.857	**0.002**
Season (breeding)[a]	-0.476	-1.026	0.151	0.112
Sex (males)[b]	0.019	-0.375	0.411	0.932
Season (breeding)[a]*sex (males)[b]	0.071	-0.485	0.494	0.750
Random				
Study	1.065	< 0.001	3.393	
Phylogeny	1.839	< 0.001	5.211	
Residual	0.008	< 0.001	0.029	
d) Eosinophils (*n* = 56, *s* = 13)				
Intercept	-3.873	-5.978	-2.199	**0.002**
Season (breeding)[a]	0.220	-0.425	0.793	0.448
Sex (males)[b]	0.251	-0.187	0.741	0.256

Season (breeding)[a]*sex (males)[b]	-0.042	-0.620	0.491	0.878
Random				
Study	1.483	0.140	3.671	
Phylogeny	2.548	0.422	5.853	
Residual	0.043	< 0.001	0.134	
e) H/L ratio (n = 110, s = 27)				
Intercept	-0.577	-1.200	0.096	0.088
Season (breeding)[a]	0.054	-0.277	0.422	0.764
Sex (males)[b]	-0.361	-0.677	-0.006	**0.032**
Season (breeding)[a]*sex (males)[b]	0.483	0.095	0.875	**0.014**
Random				
Study	0.517	0.137	0.973	
Phylogeny	0.233	< 0.001	0.777	
Residual	0.182	0.130	0.237	
f) PHA response (n = 32, s = 8)				
Intercept	0.648	0.182	1.139	**0.012**
Season (breeding)[a]	0.097	-0.140	0.320	0.420
Sex (males)[b]	0.017	-0.219	0.240	0.884
Season (breeding)[a]*sex (males)[b]	0.060	-0.239	0.309	0.664
Random				
Study	0.050	< 0.001	0.231	
Phylogeny	0.155	< 0.001	0.424	
Residual	0.034	0.018	0.056	
g) BKA assay (n = 14, s = 3)				
Intercept	-0.253	-4.987	4.873	0.864
Season (breeding)[a]	-0.195	-1.709	1.318	0.718
Sex (males)[b]	1.356	0.011	2.889	0.056
Season (breeding)[a]*sex (males)[b]	-1.594	-3.499	0.219	0.078
Random				
Study	6.441	< 0.001	22.95	
Phylogeny	5.375	< 0.001	20.10	
Residual	0.598	0.066	1.510	

[a]Relative to the non-breeding period

[b]Relative to females

Supplementary Material

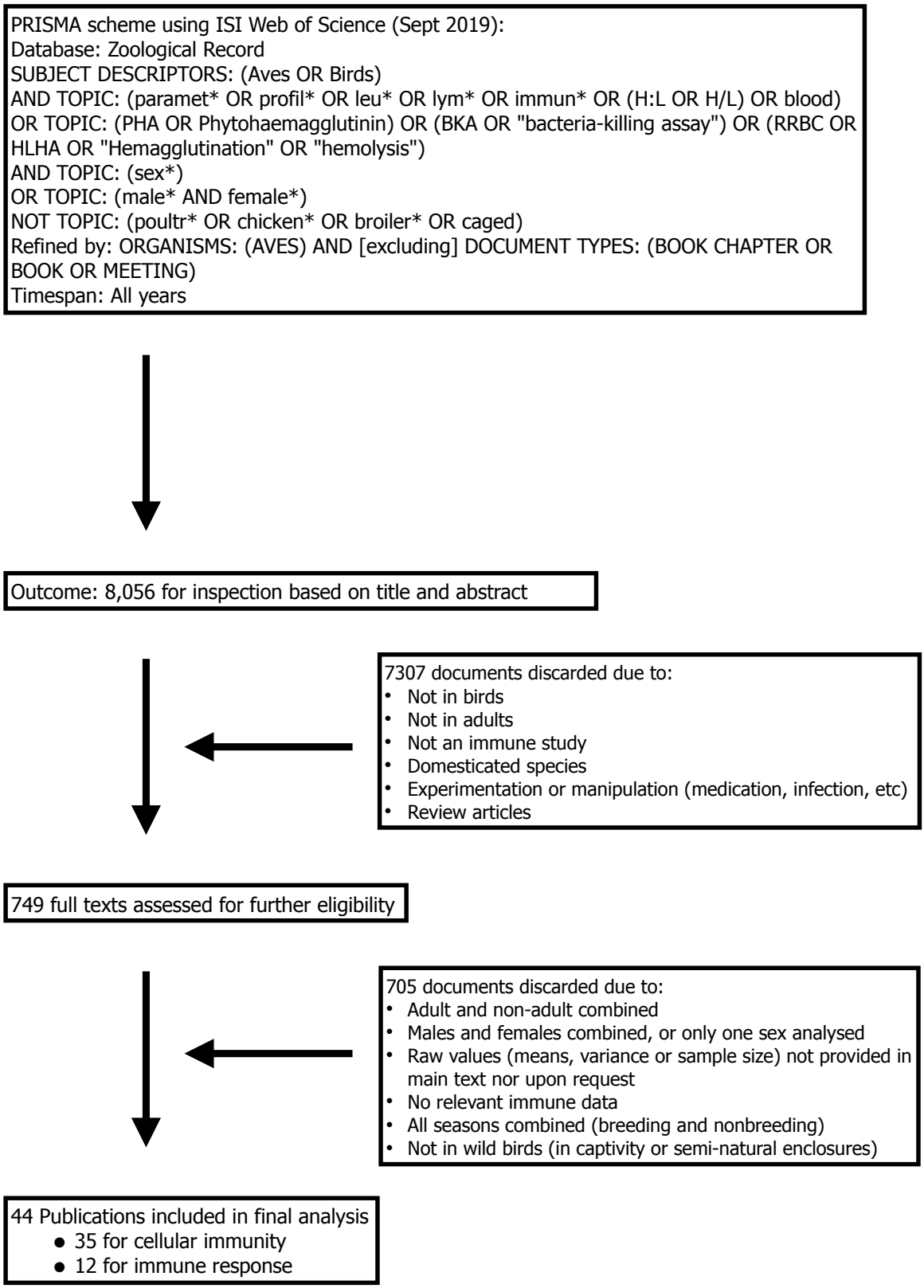
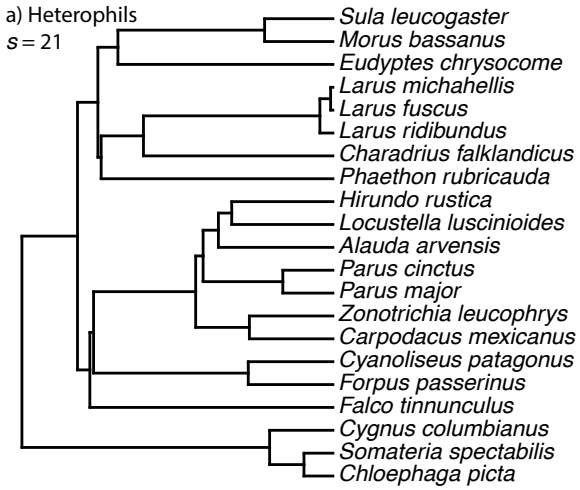
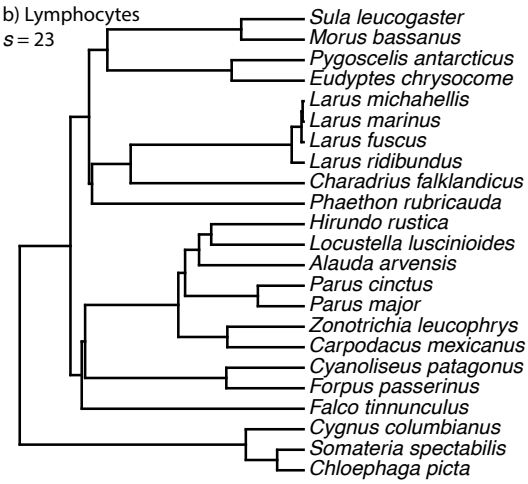
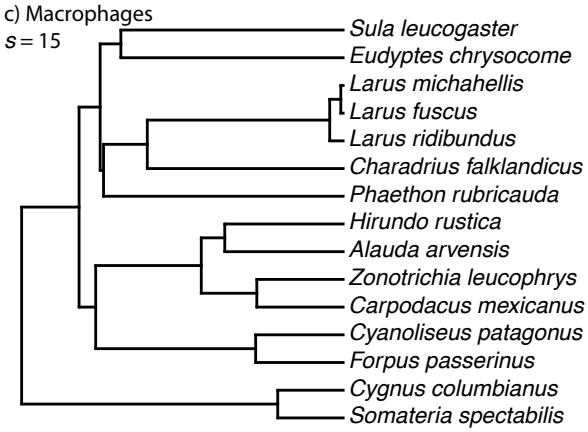


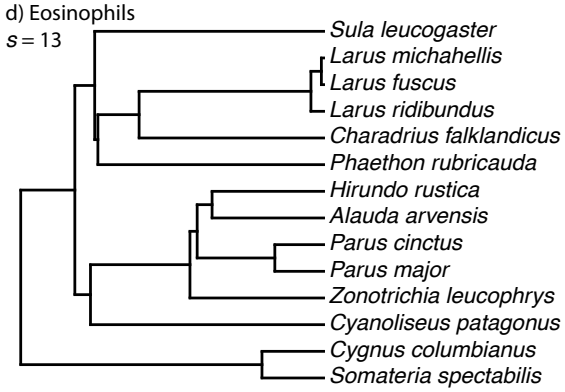
Figure S6.1 Searching parameters and PRISMA scheme followed to obtain publications used in analysis.


a) Heterophils
s = 21
Sula leucogaster
Morus bassanus
Eudyptes chrysocome
Larus michahellis
Larus fuscus
Larus ridibundus
Charadrius falklandicus
Phaethon rubricauda
Hirundo rustica
Locustella luscinioides
Alauda arvensis
Parus cinctus
Parus major
Zonotrichia leucophrys
Carpodacus mexicanus
Cyanoliseus patagonus
Forpus passerinus
Falco tinnunculus
Cygnus columbianus
Somateria spectabilis
Chloephaga picta

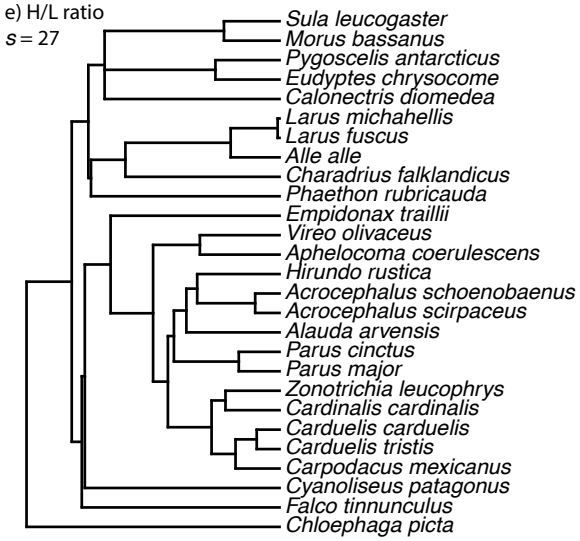

b) Lymphocytes
s = 23
Sula leucogaster
Morus bassanus
Pygoscelis antarcticus
Eudyptes chrysocome
Larus michahellis
Larus marinus
Larus fuscus
Larus ridibundus
Charadrius falklandicus
Phaethon rubricauda
Hirundo rustica
Locustella luscinioides
Alauda arvensis
Parus cinctus
Parus major
Zonotrichia leucophrys
Carpodacus mexicanus
Cyanoliseus patagonus
Forpus passerinus
Falco tinnunculus
Cygnus columbianus
Somateria spectabilis
Chloephaga picta


c) Macrophages
s = 15
Sula leucogaster
Eudyptes chrysocome
Larus michahellis
Larus fuscus
Larus ridibundus
Charadrius falklandicus
Phaethon rubricauda
Hirundo rustica
Alauda arvensis
Zonotrichia leucophrys
Carpodacus mexicanus
Cyanoliseus patagonus
Forpus passerinus
Cygnus columbianus
Somateria spectabilis

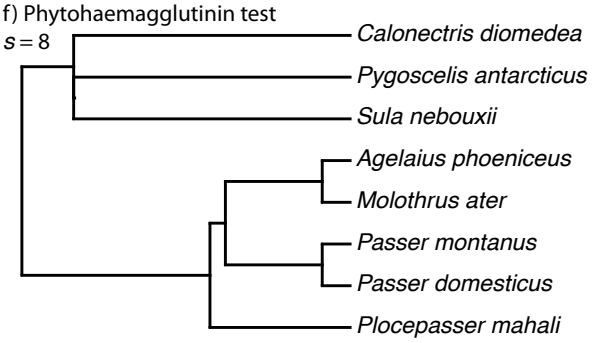
d) Eosinophils
s = 13



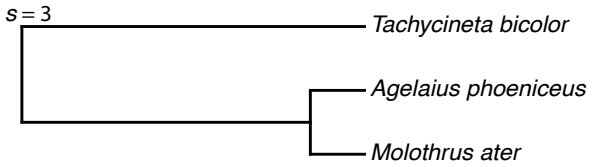
e) H/L ratio
s = 27



f) Phytohaemagglutinin test
s = 8



g) Bacteria-killing ability assay
s = 3



h) Haemolysis and haemagglutination assay
s = 3

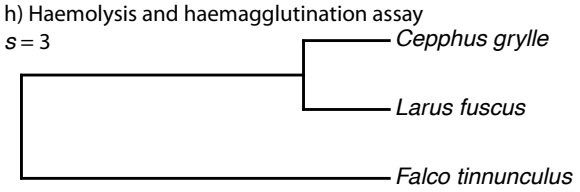


Figure S6.2 Phylogenetic trees used in meta-analyses and MCMC generalised linear mixed models. H/L ratio = heterophils/lymphocytes ratio. *s* refers to number of species.

Reference list of studies included in the meta-analysis

Cellular immunity (WBC)

1. Arriero *et al*. 2015. Variation in Immune Parameters and Disease Prevalence among Lesser Black-Backed Gulls (Larus fuscus sp.) with Different Migratory Strategies. PloS One 10: e0118279
2. Bonier *et al*. 2006. Sex-specific consequences of life in the city. Behavioral Ecology 18: 121‚Äì129
3. Boughton *et al*. 2006. An introduced generalist parasite, the sticktight flea Echidnophaga gallinacea, and its pathology in the threatened florida scrub-jay Aphelocoma coerulescens. J. Parasitol. 92(5): 941‚Äì948
4. Brousseau-Fournier *et al*. 2014. Immunologic and genotoxic profile of Northern Gannet (morus bassanus) from Bonaventure Island. Journal of Xenobiotics 4: 53-55
5. Camplani *et al*. 1999. Carotenoids, sexual signals and immune function in barn swallows from Chernobyl Proc. R. Soc. Lond. B 266: 1111-1116
6. Carbo-Ramirez & Zuria. 2017. Leukocyte profile and body condition of the house finch in tro sites with different levels of ubanization in central Mexico. Ornitologia Neotropical 28: 1-10
7. D'amico *et al*. 2017. Physiologic parameters and their response to hadling stress in a neotropical migratory shorebird during the nonbreeding season. Journal of Wildlife Diseases 53(3): 1-10
8. Dehnhard & Hennicke. 2013. Leucocyte profiles and body condition in breeding brown boobies and red-tailed tropicbirds, effects of breeding stage and sex. Australian Journal of Zoology 61: 178-185
9. Dehnhard *et al*. 2011. Do leucocyte profiles reflect temporal and sexual variation in body condition over the breeding cycles in southern rockhopper penguins? J Ornithol 152:759-768
10. Garcia *et al*. 2010. Does breeding status influence haematology and blood biochemistry of yellow-legged gulls? Acta Biologica Hungarica 61(4): 391-400
11. Giudici *et al*. 2010. Physiological ecology of breeders and sabbaticals in a pelagic seabird. Journal of Experimental Marine Biology and Ecology 389: 13-17
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13. Granthon & Williams. 2017. Avian malaria body condition and blood parameters in four songbirds. The Wilson Journal of Ornithology 129(3): 492-508.
14. Hõrak *et al*. 1998. Health and reproduction, the sex-specific clinical profile of great tits Parus major in relation to breeding. Can. J. Zool. 76: 2235-2244
15. Hegemann *et al*. Frontiers in Zoology (2013) 10: 77, & Oecologia (2012) 170: 605-618[1]
16. Jakubas *et al*. 2013. Factors Affecting Haematological Variables and Body Mass of Reed Warblers (Acrocephalus scirpaceus) and Sedge Warblers (A. schoenobaenus). Annales Zoologici Fennici 50(3): 146-157
17. Kelly *et al*. 2012. Carotenoid-based ornaments of female and male american goldfinches Spinus tristis show sex-specific correlations with immune function and metabolic rate. Physiological and Biochemical Zoology 85(4): 348-363
18. Krama *et al*. 2013. Physiological condition and blood parasites of breeding Great Tits: a comparison of core and northernmost populations. J Ornithol 154: 1019-1028
19. Krams *et al*. 2010. Effects of forest management on haematological parameters, blood parasites, and reproductive success of the Siberian tit (Poecile cinctus) in northern Finland. Ann. Zool. Fennici 47: 335-346
20. Krams *et al*. 2011. Extremely low ambient temperature affects haematological parameters and body condition in wintering Great Tits (Parus major). J Ornithol 152: 889-895
21. Kristiansen *et al*. 2006. Carotenoid coloration in great black-backed gull Larus marinus reflects individual quiality. J. Avian Biol. 37: 6-12
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27. Munoz & De la Fuente. 2003. Lymphoid distribution in the migratory gull Larus rudibundus. Comparative Biochemistry and Physiology Part A 136: 749-756
28. Owen *et al*. 2005. Habitat and sex differences in ohysiological condition of breeding southwestern willow flycatchers Empidonax traillii extimus. The Auk 122(4): 1261-1270

[1]The authors provided a dataset that was the source of the two publications cited.

29. Palacios *et al*. 2018. Cellular and humoral immunity in two highly demanding energetic life stages: reproduction and moulting in the Chinstrap Penguin. J Ornithol 159: 283‚Äì290.
30. Pap. 2002. Breeding time and sex-specific health status in the barn swallow Hirundo rustica. Can. J. Zool. 80: 2090-2099
31. Parejo & Silva. 2009. Immunity and fitness in a wild population of Eurasian kestrels Falco tinnunculus. Naturwissenschaften 96: 1193-1202
32. Plischke *et al*. 2010. Leucocytes In Adult Burrowing Parrots Cyanoliseus patagonicus in the wild, variation between contrasting breeding seasons, gender and individual condition. J Ornithol 151:347-354
33. Scott *et al*. 2010. Health Evaluation of Western Arctic King Eiders (Somateria spectabilis). Journal of Wildlife Diseases 46(4): 1290-1294
34. Sheridan *et al*. 2004. Weak association between measures of health and reproductive success in green-rumped parrotlets (Forpus passerinus) in Venezuela. The Auk 121(3): 717-725
35. Wojczulanis-Jakubas *et al*. 2015. A big storm in a small body, seasonal changes in body mass, hormone concetrations and leukocyte profile in the little auk Alle alle. Polar Biol 38: 1203-1212

Immune response tests and assays

1. Arriero *et al*. 2015. Variation in Immune Parameters and Disease Prevalence among Lesser Black-Backed Gulls (Larus fuscus sp.) with Different Migratory Strategies. PloS One 10: e0118279
2. Berzins *et al*. 2011. Sex-Specific Effects of Increased Incubation Demand on Innate Immunity in Black Guillemots. Physiological & Biochemical Zoology 84: 222-229
3. Cram *et al*. 2015. Immune Response in a Wild Bird Is Predicted by Oxidative Status, but Does Not Cause Oxidative Stress. PloS One 10: e0122421
4. Forero *et al*. 2006. Ecological and physiological variance in T-cell mediated immune response in Cory's Shearwaters. Condor 108: 865-876
5. Houdek *et al*. 2011. Innate Immunity is Not Related to the Sex of Adult Tree Swallows During the Nestling Period. Condor 113: 853-859
6. Li *et al*. 2015. Changes in phytohaemagglutinin skin-swelling responses during the breeding season in a multi-brooded species, the Eurasian Tree Sparrow: do males with higher testosterone levels show stronger immune responses? Journal of Ornithology 156: 133-141
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Table S6.1 Breeding status and time from capture to blood sampling in studies investigating cellular immunity (WBC).

Reference number	Species	Breeding status	Breeding status	Stated capture-sampling time
1	*Larus fuscus*	Breeding	Captured at nest during breeding season	Not specified
2	*Zonotrichia leucophrys*	Breeding	Captured pairs with mist nets near the nest during breeding season. Breeding status confirmed with behavioural observations	Within 3 mins after capture
3	*Aphelocoma coerulescens*	Breeding	Not specified; sampled during the breeding season. Inferred by capture dateΨ	Within 1 min after capture
4	*Morus bassanus*	Breeding	Captured at nest during breeding season	Immediately after capture
5	*Hirundo rustica*	Breeding	Captured at the gate of breeding colony (in stables)	Not specified
6	*Haemorhous mexicanus*	Breeding	Not specified; sampled during the breeding season. Inferred by capture dateΨ	Immediately after capture
7	*Charadrius falklandicus*	Non-breeding	Captured with cannon net to non-breeding birds outside the breeding season	10–232 min, mean = 105.2 (SD = 56.7)
8	*Phaethon rubricauda Sula leucogaster*	Breeding	Captured at nest during incubation and chick rearing	Within 3–10 mins after capture
9	*Eudyptes chrysocome chrysocome*	Breeding	Captured during incubation	Within 3 mins after capture
10	*Larus michahellis*	Breeding Non-breeding	Captured at nest for breeders Captured outside the breeding season for non-breeders	Not specified
11	*Calonectris diomedea*	Breeding	Captured at breeding colony	Not specified

		Non-breeding	Divided in breeders and sabbaticals (non-breeders)	
12	*Chloephaga picta leucoptera*	Breeding	Captured during chick rearing with whoosh nets	Not specified
13	*Vireo olivaceus*	Breeding	Captured by mist-netting during the breeding season	Within 30 mins after capture
14	*Parus major*	Breeding	Captured from nest boxes during the breeding season	Not specified; stated: "at capture"
15	*Alauda arvensis*	Breeding Non-breeding	Captured breeding birds 2006–2009 captured birds at moulting, autumn migration and wintering 2007–2008	Within 2–35 min, median = 5 min
16	*Acrocephalus scirpaceus* *Acrocephalus schoenobaenus*	Breeding Non-breeding	Captured at different times of the year. The last one corresponding to post-breeding, dispersal and start of autumn migration	Not specified
17	*Spinus tristis*	Breeding	Mist nets by feeders in breeding grounds. Birds were determined as breeders based on bill and plumage colours	Within 60 mins after capture
18	*Parus major*	Breeding	Captured at nest boxes during breeding	Not specified; stated: "at capture"
19	*Poecile cinctus*	Breeding	Captured at nest boxes during breeding	Not specified
20	*Parus major*	Non-breeding	Captured with mist nets by feeders in winter	Within 1 min after capture
21	*Larus marinus*	Breeding	Captured at nest in breeding colony	Not specified; handling time and lymphocyte levels not correlated P = 0.34

22	*Locustella luscinoides*	Non-breeding	Captured with mist nest post breeding and beginning of dispersal and migration	Not specified
23	*Carduelis carduelis*	Breeding	Captured during breeding season. Presence of brood patch or cloacal protuberance	Not specified
24	*Cardinalis cardinalis*	Breeding	Captured during breeding season. Presence of brood patch or cloacal protuberance	Not specified
25	*Cygnus columbianus*	Non-breeding	Captured during moulting in the breeding grounds, previous start of migration	Not specified
26	*Pygoscelis antarcticus*	Breeding	Captured at nest in breeding colony	Not specified
27	*Larus ridibundus*	Non-breeding	Captured in non-breeding grounds (Spain)	Not specified
28	*Empidonax traillii extimus*	Breeding	Captured during breeding season. Presence of brood patch or cloacal protuberance	Not specified
29	*Pygoscelis antarcticus*	Breeding Non-breeding	Captured by hand at the nest during nesting for breeders and later at moulting for non-breeders	Not specified
30	*Hirundo rustica*	Breeding	Captured using mist nets when arriving to breeding site and then during check rearing	Not specified
31	*Falco tinnunculus*	Breeding	Captured at nest boxes during breeding season	Not specified; stated: “at capture”
32	*Cyanoliseus patagonus*	Breeding	Captured at nests on cliffs during breeding season	Within 30 mins after capture

33	*Somateria spectabilis*	Breeding	Captured at the onset of nesting during breeding season	Not specified
34	*Forpus passerinus*	Breeding	Captured at nest boxes or using mist nets close to nests	Not specified
35	*Alle alle*	Breeding	Captured at nest in the colony (stated that only took breeding adults)	Within 3 mins after capture

Ψ Species breeding period was assumed according to del Hoyo *et al*. (2019) Handbook of the Birds of the World Alive. Lynx Edicions, Barcelona.

Table S6.2 Breeding status in studies investigating immune function.

Ψ Species breeding period was assumed according to del Hoyo *et al*. (2019) Handbook of the

Reference number	Species	Breeding status	Breeding status
1	*Larus fuscus*	Breeding	Captured at nest during breeding season
2	*Cepphus grylle*	Breeding	Captured when leaving nest in burrows in breeding colonies
3	*Plocepasser mahali*	Non-breeding	Captured at nest during breeding season; breeders were discarded after behavioural observations
4	*Calonectris diomedea*	Breeding	Captured by hand on their nests during the incubation, in colony
5	*Tachycineta bicolor*	Breeding	Captured from nest boxes during breeding season
6	*Passer montanus*	Breeding	Captured with mist nets and breeding status determined by anatomy and behaviour
7	*Molothrus ater* *Agelaius phoeniceus*	Breeding Non-breeding	Captured during the breeding season; only using older birds (according to plumage) to ensure they got a mate Recaptured outside the breeding season
8	*Passer domesticus*	Non-breeding	Captured just before start of breeding season. Reproductive status inferred by date of captureΨ
9	*Pygoscelis antarcticus*	Breeding Non-breeding	Captured by hand at the nest during nesting for breeders and later at moulting for non-breeders
10	*Falco tinnunculus*	Breeding	Captured at nest boxes during breeding season
11	*Sula nebouxii*	Breeding	Captured at nest in breeding colony
12	*Agelaius phoeniceus*	Breeding	Captured during breeding season while territory formation till fledging of chicks

Birds of the World Alive. Lynx Edicions, Barcelona.

Table S6.3 Number of studies, species and effect sizes used in present analyses.

Immune variable	Studies	Species	Effect sizes
White blood cells			
Heterophils	23	21	45
Lymphocytes	25	23	47
Macrophages	14	15	27
Eosinophils	12	13	24
H/L ratio	27	27	55
Immune response			
PHA	8	8	16
BKA	3	3	7
Haemolysis	3	3	3
Haemagglutination	3	3	3

H/L ratio = heterophils/lymphocytes ratio, PHA = phytohaemagglutinin test, BKA = bacteria-killing ability assay.

Model specification of Markov chain Monte Carlo simulations for generalised linear mixed models

Apart from the H/L ratio and the PHA, all our variables were proportions. Proportions were considered as success/failure (cbind(success, failure)) and the H/L ratio was log-transformed. The H/L ratio and PHA models were run with a Gaussian family distribution, whereas the rest of the models were run using a binomial family distribution (specified as 'multinomial2'). We used parameter expanded priors for the random effects (V = diag(1), nu = 1, alpha.mu = c(0), alpha.V = diag(1)), inverse Wishart priors (V = 1, n = 0.002) for the residuals and normal distributions centred on zero with large variances as fixed effects priors (default prior in function "MCMCglmm"). These priors were chosen to improve model convergence while being minimally informative (random effects) or completely uninformative (fixed effects). The models of macrophages in both full data set and subset analysis were run across 25,000,000 iterations with a thin of 20,000 and a burn-in of 5,000,000. The rest of the models were run across 1,603,000 iterations, thin of 1,600 and a burn-in of 3000. In all seven models, the potential scale reduction factor from the Gelman-Rubin test was 1.04 or lower, which is below the threshold of 1.1 indicating model convergence. Autocorrelation was also low, always below the threshold of 0.1.

Table S6.4 Number of species and number of individuals included in MCMC generalised linear mixed models of the full data set, and in the subset of data including species for which there were data from both the non-breeding and breeding periods.

	Analysis using full data set		Analysis using subset of data	
	Number of species	Number of individuals	Number of species	Number of individuals
Heterophils	21	90	3	28
Lymphocytes	23	94	4	30
Macrophages	15	56	2	14
Eosinophils	13	56	3	18
H/L ratio	27	110	7	48
PHA	8	32	5	24
BKA	3	14	2	12

Table S6.5 Sex difference and seasonal variation in white blood cell counts and immune responses in wild bird species for which there were data from both the non-breeding and breeding periods (MCMC generalised linear mixed models; *n* = total number of individuals; *P*-values < 0.05 highlighted in bold).

		95% credibility intervals		
	Post. mean	Lower	Upper	*P*
a) Heterophils (*n* = 28)				
Intercept	-1.008	-2.261	0.424	0.124
Season (breeding)[a]	0.030	-0.630	0.676	0.946
Sex (males)[b]	-0.649	-1.145	-0.242	**0.004**
Season (breeding)[a]*sex (males)[b]	0.572	-0.052	1.213	0.084
Random				
Study	0.550	< 0.001	1.852	
Phylogeny	0.592	< 0.001	2.395	
Residual	0.123	0.014	0.265	
b) Lymphocytes (*n* = 30)				
Intercept	-0.048	-1.717	1.330	0.980
Season (breeding)[a]	-0.005	-0.563	0.556	0.994
Sex (males)[b]	0.334	-0.021	0.755	0.088
Season (breeding)[a]*sex (males)[b]	-0.280	-0.834	0.283	0.352
Random				
Study	0.260	< 0.001	0.983	
Phylogeny	1.186	< 0.001	3.588	
Residual	0.119	0.029	0.231	
c) Macrophages (*n* = 14)				
Intercept	-2.679	-5.357	0.317	**0.050**

Season (breeding)[a]	-0.598	-1.538	0.223	0.142
Sex (males)[b]	0.025	-0.501	0.496	0.930
Season (breeding)[a]*sex (males)[b]	0.241	-0.943	1.325	0.674
Random				
Study	1.786	< 0.001	7.264	
Phylogeny	2.287	< 0.001	10.91	
Residual	0.035	< 0.001	0.145	
c) Eosinophils (n = 18)				
Intercept	-2.933	-4.824	-1.205	**0.018**
Season (breeding)[a]	0.410	-0.277	1.076	0.224
Sex (males)[b]	0.265	-0.292	0.838	0.346
Season (breeding)[a]*sex (males)[b]	-0.382	-1.278	0.411	0.376
Random				
Study	0.850	< 0.001	3.622	
Phylogeny	1.138	< 0.001	4.594	
Residual	0.05768	< 0.001	0.207	
c) H/L ratio (n = 48)				
Intercept	-0.553	-1.353	0.183	0.120
Season (breeding)[a]	0.066	-0.342	0.492	0.744
Sex (males)[b]	-0.389	-0.750	-0.015	**0.040**
Season (breeding)[a]*sex (males)[b]	0.556	0.091	1.070	**0.040**
Random				
Study	0.394	0.021	0.989	
Phylogeny	0.177	< 0.001	0.709	
Residual	0.212	0.126	0.306	
d) PHA response (n = 24)				
Intercept	0.805	-0.281	1.984	0.104
Season (breeding)[a]	-0.094	-0.334	0.153	0.422
Sex (males)[b]	0.091	-0.076	0.273	0.282
Season (breeding)[a]*sex (males)[b]	-0.070	-0.397	0.214	0.654
Random				
Study	0.224	< 0.001	1.021	
Phylogeny	0.531	< 0.001	1.881	
Residual	0.030	0.013	0.054	
d) BKA assay (n = 12)				
Intercept	-1.711	-6.111	2.292	0.234

Season (breeding)[a]	0.364	-1.213	1.989	0.624
Sex (males)[b]	-0.215	-1.414	1.110	0.702
Season (breeding)[a]*sex (males)[b]	1.501	-0.450	3.736	0.144
Random				
Study	3.328	< 0.001	14.14	
Phylogeny	3.707	< 0.001	11.33	
Residual	0.671	0.067	1.62	

[a]Relative to the non-breeding period

[b]Relative to females